

Genetics and the public interest

The seemingly endless studies of the ethical connotations of genetics serve a valuable role in general understanding even if the ethical problems involved are often insubstantial.

Has the whole of research been abandoned for genetics? The generality of that misapprehension is understandable. General newspapers are full of genetics, sometimes sensationally so (as when a research group at Oxford the other day was uncritically reported as claiming to be on the verge of finding "the asthma gene" and an improved treatment for familial asthma "within five years"). Then journals such as this spawn others such as *Nature Genetics* (first published this week, see facing page 460). More solemnly, the French government has briefly set aside its worries about its performance in last week's regional elections to launch legislation on biomedical ethics (see page 368). And everywhere, it seems, people who often quaintly call themselves 'ethicists' are brooding about the social implications of new knowledge in the field.

Up to a point, these broodings are welcome. Genetic knowledge has raised and will continue to raise awkward social problems; it is in everybody's interest, the research community's in particular, that they should be anticipated. But there are dangers, nicely illustrated by a report in the London *Times* on last month's conference in Houston, Texas, by Dr John Hapgood, the Anglican Archbishop of York, under the headline "The fallacy behind genetic knowledge". The supposed fallacy is that there is more to a person's identity than his or her version of the human genome, notably "our unique relationship with God". If that is a way of saying that all human behaviour is not predetermined by a set of genes, it is unexceptionable. But it would be a pity if the use of genetic knowledge came to seem to religious people to be akin to blasphemy.

That is why the brooders need a sense of history, among other things. There were genetic controversies before genes were known to reside in chromosomes (as over late nineteenth-century eugenic speculations). Ideological misrepresentations of genetic knowledge by Hitler and Lysenko were resisted, but to little avail in the short run. Present controversies centre on the prospect that techniques now practised will readily make it possible to relate some constitutional differences between people to genetic structure. Prenatal screening makes possible the avoidance of unwanted differences (but that step is not devoid of moral considerations — or controversy). But even here, cool caution is in order: Huntington's disease may be determined by a single aberrant gene, now almost (but not quite) unambiguously identifiable by known markers, but inborn characteristics determined by more than one gene are in a

different case; screening, not yet feasible, may never be on the cards.

One merit of a study of genetic screening just launched by the London-based Nuffield Foundation is that it spells out questions that can be answered tangibly, independently of extraneous issues. (The study, part of a larger project, is also unusual in that six of the nine people involved are women.) Here are the questions (out of order) and the answers they deserve.

Is prior consent to genetic screening necessary? Yes, and unambiguously. Under present law, an involuntary medical investigation is akin to a criminal assault. That is why random testing for human immunodeficiency virus (HIV) infection is not allowed except under conditions of anonymity. But the giving of consent requires the definition of a purpose with which the subject agrees and, implicitly, the exclusion of other purposes.

How should genetic information be handled and held? There is (or should be) a distinction between the storage of genomic information in the public databanks and the treatment of genetic information about individuals. The human genome projects will eventually yield a representative sequence of the human genome annotated by known variations of sequence, some of which may be more complex than has so far been imagined. But personal (as distinct from, say, ethnic) information is irrelevant and should not be accessible. Personal information gathered after informed consent should be kept by those who gather it and shared only with those to whom it refers. But there is an unmet need that data of this kind, made suitably anonymous, should be used more generally to throw light on the genetic constitution of the population as a whole.

What of confidentiality "in all its aspects"? Given procedures for informed consent restricted as to purpose, the results of genetic screening must obviously be confidential between the subject, the one who does the investigation and whoever interprets and commissions it (if different from the investigator and the subject respectively). The most common misapprehension about the use of new techniques in genetic screening is that it will be feasible to identify genetic traits simply by comparing a subject's sequence with some standard. That is not so. Even if an aberrant allele has been identified, the likelihood that it will, say, harm a future child will require an understanding of the mechanisms of inheritance. The interpretation of screening data may require an even greater degree of skill than now.



One difficulty is that those best qualified to provide it may not in future always be physicians bound by their professional duty to patients. That code may need to be extended.

Do genetic data gathered for 'legal' purposes raise ethical problems? There are two separate issues. To the extent that DNA fingerprinting is a means of identifying individuals, it is no different for other forensic techniques, fingerprinting with ink on paper or the use of blood-group categories which are but genetic markers of low resolution). So existing rules (which differ from one jurisdiction to another) should still apply. The isolation of forensic data bases from others is the best solution. The more serious difficulty is that, in the absence of information about the distribution of variant alleles in the general population, misidentification of individuals is possible (as Lewontin and Hartl have recently argued). The remedy is the still more vigorous collection of genetic data.

The implications for employment and insurance of genetic data are too often misunderstood. If it emerges that there are occupations in which people's susceptibility to unavoidable hazards (say vinyl monomer in PVC manufacture) is genetically determined, does it not make good and even humane sense that employers should seek to exclude those who are susceptible? The required safeguard is that genetic screening should not be used as a blind for more general discrimination, to which end employers should make their objectives explicit — and should be denied access to other genetic data that may be gathered at the same time. The same principles apply in applications for insurance contracts: if it is fair to weight insurance premiums against those who smoke cigarettes, should not the same apply to those with aberrant genes? The safeguards required are that insurers should not be free to cancel existing contracts in the light of new knowledge, and that liberal societies should acknowledge an obligation towards those for whom commercial insurance is declined.

Does (or will) stigma attach to those perceived to be genetically disadvantaged? The precedent of those contracting AIDS is salutary, but misleading. Prejudice against those with AIDS in the early 1980s (which still, regrettably, persists) was partly engendered by homophobia and partly by fears of infection by unproven routes. Stigmatization on genetic grounds is much less likely, especially if tight rules on confidentiality are followed. Moreover, the implied fears of genetic knowledge suppose that the construction of a person's entire genetic profile will soon be child's play, which is far from being the case.

As now, most genetic investigations will continue to be prompted by the personal anxiety of prospective parents about susceptibility to familial diseases; the promise is merely that a greater range of diseases will be open to investigation and that the quality of the data will allow more definite conclusions. Such evidence as there is suggests that those concerned welcome knowing the truth; an investigation in the 1970s among London's Greek Cypriot community of people's attitudes towards the occurrence of thalassaemia genes (partly supported by the Nuffield Foundation, as it happens) discovered that knowledge (as it has

been throughout history) is an effective antidote to unspoken anxiety. That is one reason why the present spate of new knowledge in human genetics deserves the warmest welcome. □

Not on the agenda

British science may benefit from next week's election by the impatience engendered by its studious neglect.

THE frustration of the British scientific community with the election campaign now under way may itself become a force to be reckoned with in the weeks after polling-day on 9 April. The two major parties' separate decisions not to make an issue of the state of the research enterprise and the uncertainties of higher education may be explicable by their unspoken beliefs that what Britain needs is technology and not science (as if that were possible), but the result has been a spate of letter-writing. This journal has published some of them, predominantly about academic pay (see for example page 374). The *London Times*, meanwhile, has struck a rich vein of round-robin letters, one from a distinguished group of academic researchers led by Dr Paul Nurse (University of Oxford) and, more recently, from several members of the organization called 'British Scientists Abroad'. At one stage, even the minister at the Department of Education and Science responsible for higher education, Mr Alan Hawarth, intervened with emollient reassurance.

The trouble with the argument about the condition of the research enterprise in Britain is that it cannot be conducted numerically, by comparing research support now with that in previous years, or in other countries. Notoriously, 'output' measures (usually bibliometric indices) are also misleading. As several correspondents have noted over the past several months, while there are structural difficulties in the organization of the research enterprise (the difficulty of founding research groups of critical mass, the lack of a career structure for researchers and even matters of academic pay), the true iron in the soul is that much of the British research community is demoralized, and perceives itself to be in that state. The way that recruitment to science courses at British universities has faltered suggests that the condition may persist.

If the current election does nothing for British science, the impatience that neglect engenders may itself be worthwhile. This week's letter argues that, in one respect, research institutions could do much to help themselves. That should by now be abundantly clear. Much of the present difficulty is that British universities and research laboratories brood about their parlous condition, but do less than they should to define how matters should be put to rights. Yet there is no reason to believe that research councils, the chief sources of support for basic science, are unsympathetic and every reason to expect that clear and cogent public statements of the problems that need solving would cause them to change their pattern of activity. The best outcome of the election would thus be a clear demonstration of impatience. □

NIH forms policy centre to study research ethics

- Unit will respond to emerging issues
- Outside scholars complement staff

Bethesda, Maryland

THE US National Institutes of Health (NIH) are creating a public policy centre to anticipate and help to shape the public debate on many of the controversial social, ethical and legal issues in biomedical research.

Bernadine Healy, the director of NIH, expects the centre to give NIH the intellectual resources to identify, ponder and then begin to resolve these issues before they explode into public view. But several prominent bioethicists, none of whom has been consulted about the new centre, are more sceptical of its chances for success. They applaud Healy's intentions but they question whether such an in-house centre can be independent enough to garner respect and open enough to build the consensus needed to withstand the inevitable political battles.

Healy sees the centre as "a model for what a government think tank can be". She hopes to launch it by the summer with a few million dollars from her director's discretionary fund, starting with a small permanent staff, a handful of experts on call from other parts of the institutes, and a few outside scholars and fellows from universities, industry and the non-profit world.

The centre will be located within the offices of her associate director for science policy and legislation, Jay Moskowitz. Moskowitz says that the centre will be both "a rapid response unit" to questions raised by NIH officials and a place where scholars can pursue their own lines of inquiry. The centre will also sponsor workshops and commission reports, he says, but it will not operate an extramural grants programme. Healy has assigned a major role in the planning to Sandy Chamblee, a former partner in the Washington, DC, law firm of Steptoe & Johnson, who joined the NIH in January as counselor to the NIH director and senior policy analyst (see sidebar).

Healy and Moskowitz expect the centre to wrestle with issues that could otherwise delay the progress of science, including the proper handling of genetic information, the treatment of animals in research, and the use of prisoners in clinical trials. "The era of Gregor Mendel looking at pea plants and ignoring the impact of his research on the agriculture industry is past", says Moskowitz. "NIH has moved into the nineties, and it's our job to find those emerging trends that will have major implications for policy."

As part of the Department of Health and Human Services, NIH must defer to the Bush Administration on matters of policy. But Healy believes that there is still a lot that NIH can do. A 21-page report prepared as part of the strategic planning exercise now under way at NIH points out "the aim is not to promulgate new regulations . . . but rather to provide the research community with relevant guidance and [ensure] public understanding and support for basic and clinical research".

Many bioethicists outside NIH agree that such efforts would be helpful. But they wonder if NIH is going about it in the right way, if the model is the right one, and if the centre will have to pull its punches.

"We have allowed some of these issues to become so politicized that we can no longer act", says Alex Capron of the University of Southern California, citing as an example the 1988 report on fetal tissue transplantation that was effectively ignored by the administration.

Capron was executive director of a presidential commission on ethical issues in biomedical research formed under Jimmy Carter that went out of business during the Reagan administration, and was later chairman of an ill-fated board de-

signed to advise Congress that floundered over the issue of abortion. He says that, to be effective, the NIH centre must solicit the opinions of all segments of society and, at the same time, have access to policymakers at the highest levels of the federal government.

Being seen as an internal 'job shop' would not only limit its effectiveness but also make it harder to attract leading scholars, says Albert Jonsen, chairman of the department of medical ethics at the University of Washington medical school and a member of both the presidential commission and an earlier national commission for the protection of human subjects that existed from 1974 to 1978. In addition, says Jonsen, "ethics is a communal activity, not a bench science", and he warns that "a quick response [to a problem] is often very dangerous".

John Fletcher, who was head of the bioethics programme within the Clinical Center at NIH for 10 years before leaving in 1987 to become professor of bioethics at the University of Virginia medical school, believes that the center could play an important role by re-examining those issues — including the current prohibition on the use of embryos and transplanted fetal tissue in research — first raised a decade or more ago.

"I would see it as a potential healing force", he says, "an attempt to seek knowledge and promote thoughtful debate. If the center can't do those things, it can't succeed. NIH prides itself on having the academic freedom of a university. This would be a good test."

Jeffrey Mervis

From blue-chip to white coat



WHEN Bernadine Healy, director of NIH, asked Sandy Chamblee (pictured left) to help her set up a new centre to deal with the ethical, legal, social and economic issues of biomedical research, Chamblee saw it as a chance to turn her hobby into a full-time job. Now, barely two months into her new position as senior policy analyst and counsellor to the director, the Washington lawyer says that she "could not have picked a better place to work".

Yes, she has switched from being partner in one of the city's biggest law firms (Steptoe & Johnson) to being a government employee. And yes, she will earn only a fraction of her former salary. But after 14 years as a litigator, Chamblee says that she does not miss working through the weekend on a case that lands on her desk on Friday afternoon. And she relishes the type of "complex and challenging" issues that abound in the field of bioethics.

That is what she has been doing for the past five years as a member of the board of directors of Columbia Hospital for Women, where she drafted a model consent form for embryo freezing as part of the hospital's *in vitro* fertilization programme. She has also chaired the advisory board of the Center for Humanizing Health Care within the Mediantic Healthcare Group, with which the hospital is affiliated.

While little known to the national bioethics and research communities, Chamblee has begun to make the rounds at NIH and has found the various institute directors "very interested and supportive" of the new centre. "It's an idea that needs to happen."

J.M.

Europe drafts a convention

EUROPE has taken an important step towards making bioethics a legitimate topic of political debate, as well as a universal right that governments must respect.

Meeting last week in Madrid, the chairmen of the ethics committees from the 26 countries that make up the Council of Europe adopted a draft of a European Convention on Bioethics. Participants also agreed to become members of a standing conference that would promote discussion and raise awareness of their work.

The convention — due to be ready by the end of 1993 — will consist of a framework of fundamental principles, based loosely on the European Convention on Human Rights. It will incorporate respect for human dignity, protection of individual integrity and the prohibition of all commercial agreements concerning the human body and its organs. Subsequent protocols will contain the rules for specific fields of bioethics, the first two of which will cover organ transplantation and biomedical experiments on humans.

The terms of both the convention and the protocols are expected to be quite general. While such broad wording will not be very useful as a guide to decisions on specific cases, observers believe that the documents will serve to promote the

discussion of bioethical issues across Europe. In addition, the convention is unlikely to eclipse national legislation in deciding what researchers are permitted to do.

The Standing Conference of National Ethics Committees will provide much-needed channels of communication and allow bioethical issues to be debated on a pan-European scale. Catherine Lalumière, secretary general of the Council of Europe and proposer of the conference, says that, in particular, bioethics must be discussed in the context of economic as well as scientific and legal issues.

Meanwhile, in Paris last week, three draft bioethics bills were presented to the French Council of Ministers for their consideration before being passed on to the spring session of Parliament. Few changes are expected to be made to the bills, which are couched in terms similar to the draft European convention. The French government hopes that this body of legislation, once passed, will become a reference point as its European neighbours and the United Nations discuss medical and genetic human rights. In 1983, France was the first country in Europe to set up a national ethics committee.

The three bills are sponsored by three different parts of the French government. The first bill, from Minister of Justice

Michel Sapin, concerns the law on genetic identity. It states that a person's genetic make-up should be modified only for therapeutic reasons. Genetic tests will be permitted only in scientific research, medical therapy, and judicial proceedings.

The second bill, from Health Minister Jean-Louis Bianco, concerns the use of human organs and products for therapeutic purposes. It calls for the donor to remain anonymous, and states that no financial remuneration should be made for any part of the human body. It also covers the donation of organs and tissue and medically assisted procreation, which includes anything from the use of fertility drugs to *in vitro* fertilisation. But it is silent on any diagnoses conducted before the egg is implanted in the womb. Such procedures have been prohibited in France since 1986.

The third bill is from Hubert Curien, minister of research and technology. It deals with the computerization and management of health and genetic data.

Also last week, in London, the Nuffield Council on Bioethics announced that a panel has been formed to examine genetic screening. The group will look at the techniques involved, their benefits and difficulties, and such ethical issues as the handling and holding of information and consent to being screened. The panel will report to the council — which has no legislative power — within 18 months.

Ian Mundell

JAPAN BIOETHICS

When silence isn't golden

Tokyo

ON a list of thankless jobs in science, being chair of a meeting in Japan on the ethics of human genome research must rank near the top. If you doubt it, just ask Norio Fujiki.

Fujiki, a member of the faculty at Fukui Medical School on the Japan sea coast, received a grant for just such a conference from one of the leaders of Japan's Human Genome Project. But the two-day seminar last month at Fukui confirmed Fujiki's worst fears: his colleagues had so little interest in the subject that they spent the first day discussing the scientific and clinical aspects of the project and then left before the talk turned to ethics.

Kenichi Matsubara provided funding for the conference by assigning 1 per cent of his \$3-million a year grant from the Ministry of Education, Science and Culture to a small group led by Fujiki. Unlike the US National Institutes of Health, which is devoting some 5 per cent of its \$103 million genome budget this year to research on a variety of ethical, legal and social issues, Fujiki is almost alone in receiving money to examine these questions. Nearly all of his \$30,000

grant was spent on organising the seminar, which was held for the second time this year.

To attract life scientists to Fukui, Fujiki devoted the first day of the seminar to an overview of genome research and to scientific presentations on clinical applications of medical genetics. The second day examined social, legal and ethical issues. But, much to the dismay of Fujiki and other bioethicists, most of the life scientists among the speakers and audience did not return for the second half of the seminar.

In fairness, Fujiki points out that some of the speakers, including Matsubara, were busy negotiating with the government on funding decisions that had to be made before the end of the fiscal year on 31 March. But he admits to being "so disappointed" with the poor attendance on the second day, and he blames himself for failing to balance the programme better.

Daryl Macer, who teaches a course on bioethics at Tsukuba University, says that many of the scientists who made presentations on the first day were incapable of communicating well with their audience.

He says that their highly technical talks went over the heads of the 150 people, many of them nonscientists, who attended the meeting because of its supposed focus on ethics.

Apart from Fujiki, the only other group with government support to examine such issues is that led by Tadami Chizuka, professor of European history at Tokyo University. Chizuka and a colleague from Tsukuba University received a grant of ¥5 million (US\$37,000) last year from the Ministry of Health and Welfare to translate French and German legislation on the human genome project into Japanese.

Japanese scientists are reluctant to discuss ethical issues because they lack expertise on such matters, according to one genome researcher. They also worry about being viewed as too political if they become involved.

The medical community also has shown little interest so far in the subject. Although there are bioethics committees in nearly all of Japan's hospitals and university medical departments, Chizuka says that they are not looking at issues raised by the human genome project because gene therapy has not yet reached the point of practical applications.

David Swinbanks

How much green in the greenhouse?

Washington

WHEN there is doubt, speak out. Or hire some researcher to do it for you. Uncertainty over how greenhouse warming will affect the climate — and the public's craving for answers — is providing a lucrative market for a few outspoken proponents and sceptics who are willing to go beyond the usual caveats and caution of science into outright advocacy.

It will be many years before science can truly understand the relationship between rising levels of carbon dioxide and other greenhouse gases and global climate. But that is not stopping the public calls for limits or curbs on present-day emissions. With such potentially costly policies on the line, both industry and the environmental groups seem to be convinced that money spent on a few well-spoken researchers goes a lot further in winning over the public than does money for research itself.

These researcher/advocates have become so visible, in fact, that rumours abound of the big money that can be made by jumping aboard the lecture circuit. One version paints industry as so eager to discount the existence of global warming that it is dangling research grants and fat fees in front of any scientist willing to be a public sceptic. Another version accuses the environmental movement of sparing no expense in its quest to convince the public that global warming is already here, and certain to grow worse unless dramatic steps are taken to curb the world's production of greenhouse gases.

Last month, for example, a newsletter produced by researchers at the University of East Anglia Climate Research Unit in Britain, *Tiempo*, put the potential profits of public scepticism at \$10,000 a month for researchers who live in the United States and "have friends, or make them, in the fossil fuel industry." In turn, sceptics say that industry pales next to the environmental groups — the "biggest lobby in America" — when it comes to sponsoring friendly researchers.

The truth is that scientists on either side of the debate are collecting sizeable fees for offering their versions of the truth. Contrary to the rumours, however, no one appears to be getting rich. Interviews with several of the scientists who travel the lecture circuit found that none earned more than \$30,000 last year.

Greenhouse sceptic Patrick Michaels, a climate researcher at the University of Virginia, made 43 speeches last year and earned \$25,490. Another sceptic, Robert

Balling of Arizona State University, made about 15 speeches for which he says he was paid slightly more than \$10,000.

On the 'believer' side, Stephen Schneider, a climatologist at the National Center for Atmospheric Research at Boulder, Colorado, made about a dozen paid speeches last year and earned about \$30,000. And Michael Oppenheimer, a staff researcher at the Environmental Defense Fund, made about 50 paid and unpaid speeches last year. He says he has "netted less than \$15,000" from speeches given during the past three years.

Other rewards for advocacy go beyond lecture fees. The Pacific Research Institute for Public Policy and the Cato Insti-

sceptics show up more in the press than they do in the scientific community. Although about 70 per cent of researchers (members of the American Meteorological Society and the American Geophysical Union who were sampled by Gallup pollsters) thought that global warming was under way, according to a survey by the centre, only about half of the scientists quoted in newspapers such as the *Washington Post* and the *Wall Street Journal* held that view. PR, in short, works.

"I think industry is making a concerted effort to confuse the public on this issue," says Daniel Lashoff of the Natural Resources Defense Council. "They're sowing the seeds of confusion by paying hired



Two sceptics and a believer: Special-interest backing helps Balling, Michaels and Schneider (left to right) travel the greenhouse lecture circuit.

tute, two think-tanks that defend the free market, are publishing books by Ballings and Michaels. And Michaels' resume lists a one-year \$50,000 grant from an anonymous source — someone, presumably, who approved of his sceptical line.

Whatever the motives of their sponsors, most of these researchers describe their constant touring and advocacy as primarily a labour of love (or duty). The work, they say, pays just a fraction of what a scientist can earn as consultant for the pharmaceutical industry or by providing expert testimony in court.

Although all the researchers interviewed emphasize that they charge nothing beyond expenses for many — sometimes even the majority — of the speeches they make, the fees add up to quite a tidy sum. The best-known greenhouse speakers such as Schneider and Michaels can make \$3,000 or more per lecture. "I don't think the [sceptics] are in it for the money any more than I am," says Schneider. "But if the money's there, we'll take it."

Who is winning this war of words? The Washington-based Center for Science, Technology and Media has found that the

guns to take pot shots at the science and scientists, rather than arguing policy."

Researchers who partake in the "travelling dog-and-pony shows" — the seemingly endless series of lectures, debates and panel discussions before such nonscientific groups as the United Ski Industries Association and the Council of Industrial Boiler Owners — defend it as a necessary evil brought on by environmental politics. Decisions are being made now, even without scientific consensus, they say. Better that people be exposed to a little one-sided science than none at all.

By paying outspoken researchers to spread the word with a scientific seal of approval, Schneider says, both industry and environmental groups are simply ensuring that their side will be heard: "I don't think the money is making these guys say what they do; they were saying the same thing before the money came along. All it's done is leverage the probability that they will get in the paper."

Sometimes, Schneider says, researchers must become advocates. And if it means making some money on the side, so be it.

Christopher Anderson

Smaller, but still controversial

Washington

CHEAPER and split into smaller parts, this year's version of the Earth Observing System (EOS) of 1992 resembles its predecessors only in its ability to generate controversy. Although the US Congress may feel better about the cost, one-third lower than the original \$16,000 million, critics say that the current plan to launch a series of small- to medium-sized orbiters late in the decade puts expediency before science and is an invitation to delay.

The idea for EOS came not from the research community but from officials at the US National Aeronautics and Space Administration (NASA). When outsiders complained that it was just another mega-engineering projects, NASA won support from the US Congress and much of the scientific community by making it the star of its eco-friendly Mission to Planet Earth programme.

But EOS remains suspect because of its origins. Last fall, Congress decided to trim NASA's sails and told the agency that the project could cost no more than \$11,000 million. That limit — and some less-than-

subtle language in the appropriations bill — forced NASA to redesign EOS.

In December, then NASA administrator Richard Truly announced that the project would be redesigned as a series of smaller platforms and staggered launches. Those details were revealed in March in a submission to Congress. Instead of two large platforms, both huge satellites about the size of the Hubble Telescope, EOS now will consist of six smaller mission that will fly on intermediate-sized boosters like the tried-and-true Atlas IIAS series.

Although the original EOS programme was to focus on broad issues of global changes, including ozone depletion, Earth physics and stratospheric chemistry, the new version concentrates on climate change. There are only 17 instruments instead of the original 30, with six deferred until the middle of the next decade at the earliest.

Most researchers have welcomed the move to smaller platforms. With the flawed Hubble telescope and the tragic Challenger space shuttle in mind, they point out that smaller platforms have less at stake if one

part goes wrong and allow scientists to modify later missions to correct deficiencies. But they are less happy about the order of the missions, which some say is a result of convenience rather than a response to the most pressing climate change questions.

As EOS now stands, the first platform (scheduled for 1998) will be a medium-sized satellite known as EOS-AM. Its Sun-synchronous orbit will keep it in perpetual morning, where it can observe the Earth when cloud cover is at a minimum. That platform will be followed by two smaller missions that will look at oceanic biomass and atmospheric aerosols. Two years later comes EOS-PM, another medium-sized probe that will observe the Earth in the afternoon, a time best suited for meteorological forecasting.

NASA argues that EOS-AM should fly first because scientists are most in need of the data from its instruments, including an infrared surface imager known as ASTER. (NASA gives models of earth biology a score of two on a scale of ten.). No one argues with that characterization, but there is concern as to whether EOS-AM will be able to answer any important questions soon.

"The ecologists don't know anything about their models," says one researcher. "You dump all this data on them and they won't know what to do with it."

Meanwhile, the answer to the question that all the policy-makers have been waiting for — is global warming happening, and if so, how fast? — seems likely to have to wait until the next century. That's when EOS-PM, which looks at atmospheric dynamics and global moisture, will start sending back data.

Researchers blame politics and tight funding for the apparently skewed priorities. EOS-AM benefits from the fact that ASTER is being built and paid for by the Japanese and is likely to be ready on time. (In fact, NASA was able to move the first EOS launch date forward by six months by going with EOS-AM.) At a time when NASA cannot afford delays or cost overruns, the benefits of an on-time, low-cost start to EOS are considerable.

"If you're going to launch just one, I'd say EOS-PM", says Dennis Hartmann, a University of Washington atmospheric researcher and chair of the EOS investigator's atmospheric panel. "But when you look at the practical realities — budget and schedule — you're drawn towards AM."

Accompanying the debate over the proper order is fear that the growing US deficit will stretch out the timetable for later missions. One congressional aide warns that EOS-AM could be the only mission launched in the next ten years. With EOS-PM and its compatriots stuck on the ground, such a delay could leave everybody but the ecologists up in the air.

Christopher Anderson

US/RUSSIA COLLABORATION

Russians want business, not charity

Washington

RUSSIAN scientists lack a great deal these days, but they have lost none of their pride. Despite having to cross 5,000 miles, the message from senior science policymakers came across loud and clear during a two-hour video conference last week with members of the science committee of the US House of Representatives: Russian science wants a handshake from the United States, not a handout.

"Russia has something to offer you. We're not an under-developed country", Boris Saltykov, minister of science, advanced education and technology policy, told Representative George Brown (Democrat, California), who chairs the science committee. "The assistance that we're seeking must be one of mutual benefit. Although it could help alleviate our economic hardship, it could also provide knowledge that you in the United States are now lacking."

The teleconference was the third in a series of televised discussions, and the first international linkage, between the House Committee on Science, Space, and Technology and the scientific community. The hearing featured Saltykov and four other prominent Russian science administrators: Yuri Osipov, president of the Russian Academy of Sciences; Andrei Gonchar, first vice president of the academy; Yevgeniy Velikhov, an academy

vice president, director of the Kurchatov Institute and a leading figure in disarmament efforts; and Yuri Koptev, director of the Space Research Institute and salesman to the West of the fruits of the former Soviet space programme. The communications system performed flawlessly.

The Russian delegation acknowledged the serious problems facing all areas of science and said repeatedly that Western cooperation could help to keep laboratories intact. But the most serious consequence of a possible brain-drain, they said, is not the loss of existing talent but the fact that it may not leave enough people to teach the next generation of scientists.

Russian is having "tremendous problems" converting from a defence-oriented economy to a civilian one, Velikhov said. And his colleagues suggested that the wrenching cultural changes needed for Russian science to thrive in the post-Soviet era may pose the biggest barrier to success.

"Conversion is more than just factories", said Osipov. "We also have to convert people's way of thinking. Right now we have thousands of scientists from the academy who had been doing military work and now have nothing to do. They are no longer being supported by the academy, and nobody knows what they are going to do next."

Jeffrey Mervis

Grassroots consortia go back to the basics

Washington

WITH little fanfare, a small California software company is proving that it is possible to create innovative and profitable industrial research consortia without massive government subsidies or cumbersome bureaucracies. At the same time that big industrial consortia such as



Sematech and the Microelectronics and Computer Technology Corporation (MCC) are wracked by internal dissension and losing members, Biosym Technologies, a company based in San Diego that specializes in molecular and chemical modelling software, has started four consortia involving more than 100 industrial members and put products on the market.

Biosym has succeeded by sticking to the basics and steering clear of its members' proprietary secrets. By focusing on basic molecular modelling tools with broad application, the company has managed to find a 'generic' niche that is still state-of-the-art. Another part of the formula for success is to let its own employees — not researchers on loan from its member companies — do the work. That approach provides a buffer between the competing partners and ensures that no proprietary technology is leaked. Members meet every nine months to vote on research directions, but otherwise they simply put in their money and wait for results.

This grassroots approach to consortia has won nothing but praise. The products keep coming, and members say that they are getting what they want at a fraction of what it would have cost them to do it themselves. Last year, Biosym had total revenues of \$23 million and increased its staff by 54 per cent, becoming the 20th fastest growing US high-technology company.

Biosym's style of narrowly-focused, do-it-yourself consortia appeal to companies grown wary of costly, government-subsidized research collaborations with fuzzy research aims and power struggles between members. (Sematech, for example, operates on an annual budget of \$200 million, half from the federal government and half from its 14 industrial members.) Cray Research has started a similar consortium to develop chemical modelling software for its supercomputers, and dozens of university research groups are winning industrial funding by setting up their own small consortia to take on specific research projects.

The aim of all of these consortia is to make the best use of scarce research dollars by addressing common problems. But some do it better than others. Sematech has had a hard time finding such problems — it initially intended to research semiconductor materials, but was forced to shift to manufacturing technology when its members got nervous about jointly working in such a competitive field. In contrast, Biosym started on molecular and chemical software tools and has never looked back.

Its four consortia — polymers, catalysts, potential energy functions and a materials project that will start in July — each have about 15 full-time programmers. The polymer consortium charges each of its 51 industrial members about \$80,000 a year. In exchange, it gives them new software every nine months and asks them to vote on the next project. The members get one year of exclusive access to the software, after which Biosym can sell it to anyone.

Universities may join by paying 15 per cent of the commercial rate, but they have no vote in setting the direction of research. Any company that joins late must pay all back fees.

Other consortia have similar ground rules. Cray's two-year-old UniChem project has fewer members (five) and charges more, but otherwise the meet-and-steer process is the same, as is the promise of a period of exclusive use.

Industrial members say that these consortia save them from having to develop their own software but still allow them to tailor the final product to their specifications. But while basic atomic and molecular modelling tools have applications ranging from drug design to catalyst creation and materials research, not many other technologies can make the same claim.

"Anything other than software gets proprietary real quick," says George Fitzgerald, a member of the Cray Research UniChem team. When software goes beyond the basic modelling tools it enters an area where members want different features and tend to fight — or quit — if they feel that they are not getting what they need. That, of course, is something Sematech and MCC have learned only too well.

Another advantage of small consortia over in-house research teams is their ability to search the world for related technology. Biosym's academic connections have unearthed dozens of specialized algorithms and software tools, many of which were available essentially without charge. The consortia has also succeeded in finding good software that was nevertheless lying fallow because of its clumsy user interface or unusual

hardware requirements.

"Biosym's been able to find bits of software from around the world that were essentially unusable," says Warren Knox, senior research manager at Dow Chemical Company, a member of the polymer consortium. "They've collected it, organized it and made sure the connections are seamless." Encouraged by Biosym's handling of 'found' software, Dow even donated an in-house molecular structure program of its own — and was delighted to see the software given a easy-to-use interface and worked into the next consortia software package.

Members of the consortia are not deterred by the thought that their competitors will have access to the same technology. "The proprietary concern is balanced with the knowledge that there are times when you just can't afford to go it alone," says Thomas Rauechle, UniChem product manager. "And the companies only have to say what they want, not what they intend to do with it."

Rather than worrying about their competitors, members of the consortia value the collaborations as a sort of neutral zone where they can discuss shared problems without the usual trade-secrets caution. "It lets us talk to our competitors, which we wouldn't do otherwise," says Knox.

Apart from those working on software, most of the small consortia are based around university groups (such as catalysis consortia at the University of Delaware and the University of Chicago) and unique problems. For example, a number of chemical companies who are looking for alternatives to the ozone-killing chlorofluorocarbons (CFCs) have joined together to do toxicity testing on the new compounds.

But industry's romance with small consortia may not last forever. The number of problems that are generic enough to allow fierce competitors to join amiably are limited. And some industries may be at capacity.

"Companies are getting consortiumed to death," says Kenneth Peddicord, dean of engineering at Texas A&M University. That development is not all bad, he adds: the competition for industry dollars "has sharpened the research that's going on inside" the consortia.

Industrial researchers say that such willingness to shift focus quickly to suit corporate sponsors proves that small consortia are staying flexible — a trick their big rivals have yet to learn. As long as industrial research funding remains tight, they say, small, independent consortia will continue to make sense as a way to perform high-quality research on the cheap.

Christopher Anderson

Panel calls for technology company

Washington

CALLING for a \$5,000 million "civilian technology corporation", the National Academy of Sciences last week recommended that the US government match technical and manufacturing advances in other countries by funding 'pre-commercial' research and development (R&D).

Although the Academy report* acknowledges that the US high-technology industry remains relatively healthy on such measures as productivity per capita and trade balance, it points out such warning signs as a decline in R&D spending and the sluggish transfer of technology from government research laboratories to industry.

Market forces and existing ways to transfer technology are not enough to keep the United States competitive, the report concludes; the government must intervene. However, the right sort of interven-

tion remains controversial. After raising and dismissing such solutions as having existing agencies spend more on pre-commercial R&D (the disadvantage: no real mandate for change) and creating a new agency modelled after a civilian Defense Advanced Research Projects Agency (too vulnerable to politics), the NAS panel reluctantly settled on an independent technology corporation. Although it, too, could be influenced by politics and hindered by the absence of clear market signals, "it was the least bad solution," said Harold Brown, the chairman of the panel and a former Secretary of Defense.

The report also calls for an 'Industrial Extension Service' at the US Department of Commerce, modelled after the venerable extension programmes of the US Department of Agriculture. The panel recommended that a few of the 700 federal

laboratories be converted into facilities that would demonstrate federal technology to industry.

At the heart of the report is its call for a civilian technology corporation. Funded by a one-time, \$5,000 million appropriation and managed by a politically appointed board, the company would support R&D by funding industrial research. Proposals would come from industry itself, the panel was careful to note; eager to avoid the stigma of 'industrial policy', the report argues that such 'bottom-up' economic support would not interfere with market forces.

Real-world examples of such a corporation are scarce. At a press conference last week, Brown cited the Communications Satellite Company (Comsat) and the ill-fated Synthetic Fuels Corporation. But Comsat concentrates on telecommunications, and the synfuels company went under in 1986 after oil prices dropped.

How the corporation would work is still an open question. Start-up funding, loans and joint ventures with industry are all possibilities, said Brown, adding that "we'll leave it to its directors".

The idea itself was enough for US Sen. Ernest Hollings (Democrat, South Carolina), who had asked for the report. At a hearing last week, Hollings announced that he would introduce legislation to create such a civilian technology corporation. But its chance of passage, concedes one staff member, are "bleak".

Christopher Anderson

**The Government Role in Civilian Technology: Building a New Alliance, National Academy of Sciences, 1992.*

OCEAN MONITORING

Economic crisis squeezes data

London

A LACK of money to operate a fleet of Russian research vessels is undermining an international system that monitors the salinity and temperature of the upper layer of the world's oceans. In addition, the rise of capitalism in the former Soviet Union is expected to drive up the future cost of operating such a system.

A quarter of the 155 ships that contribute data to the Integrated Global Ocean Services System (IGOSS) are maintained by the states of the former Soviet Union. But only four of the 38 research and commercial vessels that participated in IGOSS have been active since the coup last August that led to the final dissolution of the Soviet system. The rest of Russia's 120-ship research fleet is similarly grounded.

Run jointly by the World Meteorological Organisation (WMO) and the Intergovernmental Oceanographic Commission (IOC), IGOSS relies on data collected voluntarily by the crews of research and commercial vessels all over the world. The information is fed into the WMO Global Telecommunications System, where it is picked up by meteorologists and the few oceanographers doing 'real-time' research. After a month the data are archived for longer-term studies.

"The Soviets were a major contributor of this type of data, and we saw a drastic drop in the data flow around the time of the coup," said Tim Wright, coordinator of IGOSS operations. "They have an excellent oceanographic fleet, which is now just sitting at pier. If not used, the ships tend to go downhill fast. It's a pretty grim situation."

The ships are operated by a number of

agencies, including the Russian Academy of Sciences, the Committee for Geology Research and the Russian Hydrometeorology Committee. In some cases ownership is unclear, as with a dispute between Russia and Ukraine involving jurisdiction over the Black Sea fleet.

Alexander Metalnikov of the hydrometeorology committee says that he hopes the financial difficulties can be resolved and that money can be found soon to operate the vessels. But he acknowledges that efforts to find sponsors for the committee's 24 research ships have so far been unsuccessful. Rehabilitating as few as five would be useful, he says. A typical 1,500-ton research vessel costs about \$10,000 to \$20,000 a year to operate.

Compounding the problem is the fact that the owners of Russian commercial ships that participated in IGOSS now want to be paid for their work. Under the old, state-run system, their contributions were provided at no cost to the scientific community.

The old order of the Soviet Union was singularly good at organizing the sort of large-scale operation that supported IGOSS, and its ships contributed a large number of datasets from all over the world. In particular, they covered areas in the northern seas and in the Pacific that were seldom visited by other nations, and their crews were particularly diligent in taking salinity measurements.

Salinity data are important in predicting the way masses of water behave when they meet. They are also important in understanding how fresh water flows into the sea and the evaporation cycle.

Ian Mundell

BIOTECH PATENTS

Australia says yes

Sydney

AN Australian parliamentary committee says that the government should permit the patenting of live organisms and that any objections to genetically modified organisms should be dealt with by other means. The report, by the Standing Committee on Industry, Science and Technology, is not expected to be challenged by the rest of Parliament. Its significance is heightened by the fact that Australia is the only country so far to approve such a live organism for general release.

The committee's report concentrates on setting guidelines for the conditions under which such organisms should be used. It recommends that existing, semivoluntary approved processes be strengthened and given the force of law. It also calls for a formal mechanism to obtain approval for the release of both live organisms and those that are by-products of such genetic engineering.

Mark Lawson

A speeded-up plan to spread the wealth

Paris

THE French government has accelerated plans to decentralize its research establishment by announcing the transfer over the next three years of more than 2,500 researchers from Paris to regional centres. An additional 2,000 scientists will leave the capital by the end of the decade as part of an effort by the government to distribute public-sector jobs across the country.

The purpose is both to loosen the iron grip of Paris over the conduct of research and to stimulate regional development. Although the greater Paris area, known as the Ile de France, contains less than a fifth of the country's population and only a quarter of its student population, it accounts for more than half of the research sponsored by the government.

The decentralization will be felt by all research agencies. In particular, the Centre National de la Recherche Scientifique (CNRS), the nation's major research organization, is to transfer some 2,700 staff to various regional centres by the end of the decade. That shift will reduce the proportion of its staff in Paris from 52 per cent at present to only 40 per cent by 2000. The cost has been estimated at FF1,300 million (US\$220 million). CNRS officials say that the shift will improve research and that they will not 'decentralize for the sake of decentralization'.

The scientific community has so far reacted positively, in contrast with the protests that have accompanied similar transfers in the past. Images of the en-

forced deportation of unhappy masses of white coats have faded, replaced by the recognition that the changes will be more subtle.

Nearly two-thirds of the new positions are expected to be filled by the movement of individual scientists to existing research establishments elsewhere. In most of the remaining cases, according to officials, researchers will have the chance to build their own teams by moving into new or remodelled and expanded institutions.

Although some of the dislocations will be jarring, Martine Bentaboulet, assistant director of regional development at the CNRS, says that the closing of laboratories in Paris and the forced transfer of their staffs "will occur only in a minority of cases". The CNRS says that it is still discussing which laboratories will be closed.

The stepped-up plans to decentralize French research follows on a deliberate effort to encourage growth outside Paris. For several years, the CNRS has allocated two positions to the regions for every one in the capital. Although officials argue that admission is still based on scientific merit, researchers have complained that candidates willing to set up shop in regional centres have received preference over those who wish to work in Paris. And the application form for admission to the CNRS requires candidates to nominate regional laboratories for two of their three choices.

In 1990, the CNRS extended its decentralization policy beyond staffing to the

allocation of resources for new laboratories. That year the Institute of Biology and the Institute of Microelectronics were created in Lille, followed in 1991 by the Institute of Particle Physics in Marseilles and the Institute of Structural Biology in Grenoble. This year a materials laboratory will be built in Bordeaux, and the Maison des Sciences de l'Homme in Toulouse. Each of the new centres reserved space for new research teams from the outside.

Government officials concede that the plan cannot be successful without support from researchers. The improvement of living and working conditions is essential to entice people to leave Paris. Researchers will want proof that the new laboratories are well-equipped, that there are opportunities for promotion and that the overall scientific environment is sound. And there must also be acceptable housing, good schools and job opportunities for spouses.

It is too early to know whether the government will have the resources to carry out its ambitious plan. An agreement last autumn created a special fund for researchers, but so far the fund is empty.

The decentralization plan rests on the concept of creating research 'poles'. Such poles are loose federations of laboratories within a region that, together with local universities and industry, provide a critical mass of research. The CNRS hopes to conduct half of its regional research at seven such centres, with the rest of its funding spread over 16 cities and towns. Putting mathematicians in the Marseille-Luminy area, as well as in Nice, Bordeaux and Lyons, is expected to strengthen interaction with physicists, computer scientists and mechanical engineers in those areas. Similarly, physicists, chemists and biologists will coexist at the Institute for Structural Biology in Grenoble, as well as at similar centres in Marseilles and Montpellier.

There will also be a shift within the Ile de France itself from the centre of Paris towards the outskirts. For example, installations in the humanities and social sciences will be closed, and an archaeology facility and a science centre will be built in Nanterre and Marne la Vallée.

The decentralization will also affect the CNRS administration. In 1990, 12 regional councils were created to work with local authorities, and a staff of 1,070 scattered around seven sites in Paris will be trimmed to 700 and consolidated at one site. More than 200 administrators who direct interdisciplinary programmes will be shifted to the regions, and about 100 will return to the laboratory. In 1995, the entire staff of the Institut Information Scientifique et Technique (INIST) will be transferred to Nancy in the east.

David Bakewell

LEPROSY VACCINE

India questions Venezuelan data

New Delhi

A PUBLISHED report from Venezuela that questions the effectiveness of an anti-leprosy vaccine has stirred debate in India about the fate of an extensive clinical trial using the vaccine. Although some Indian specialists believe that the trial, sponsored by the World Health Organisation (WHO), should be halted, the government says that last month's report in *Lancet* (339, 446-50; 1992) is not relevant to India and that the ongoing trial will continue.

"Obviously the WHO vaccine is of no use to India", says Mahadev Deo, director of the Cancer Research Institute (CRI) in Bombay. "Continuation of the trial would be unethical."

But officials from the Indian Council of Medical Research (ICMR) say that the differing epidemiology and geographical variation in mycobacteria in Venezuela could affect the efficacy of the vaccine. "We don't know if the vaccine will succeed in India", says S.P. Tripathy, chief of the council. "But we cannot stop the trial

because it did not do well in Venezuela."

The WHO vaccine is prepared from killed *Mycobacterium leprae* that are harvested from infected armadillo. In the Venezuelan trial, which was begun in 1983, some 29,000 subjects received either bacillus Calmette-Guerin (BCG) alone or with *M. leprae*. The scientists found no evidence that the combined vaccine offered better protection against leprosy than the BCG vaccine alone.

However, an accompanying editorial in *Lancet* warns that "interpretation of the result is not straightforward". The *M. leprae* antigens provided some additional protection against the disease, the journal notes, and the findings themselves constitute the first demonstrated effect of BCG on leprosy in the New World. The WHO vaccine is being tested in Malawi as well as India, both of which have rates of leprosy far higher than in Venezuela. The Indian trial began in January 1991 and involves 50,000 subjects.

K.S. Jayaraman

Managerial inadequacies

SIR — Howard Morris is certainly not alone in his perception of the 'managerial inadequacies' of those who are entrusted with running Britain's universities (*Nature* 356, 10; 1992). It is nonetheless disappointing that his complaint should focus so heavily on salaries. To the extent that Morris's case applies to the research function of universities, there is in fact good reason to believe that the present difficulties are not primarily related to the level of remuneration. We would suggest that the primary failure of the universities lies in their unwillingness to restructure, in order to face the demands of modern research and postgraduate education. Like much of our formerly world class industrial base, the universities seem to believe that more of the same will do. Well, it will not. However important it may be that this country should spend more of its gross national product on research, the necessity of increasing the undergraduate population substantially, while maintaining a first-class research and postgraduate training programme, deserves to be met with more imagination and realism than a simple demand for

more cash.

The universities must also begin to take a proper interest in the training, development, assessment and career progression of its research staff (a point touched on by Morris and another recent correspondent, *Nature* 355, 292; 1992). These people are after all its main asset. Recent proposals from the Association of Researchers in Medicine and Science (*Careers in Research*) call for a much greater role for management of the research enterprise and the employment of research staff. The proposals may not provide a complete answer to the problems, but they do suggest some solutions to important issues that the universities have so far failed to address.

When faced with administrative and management problems, the conservatism and lack of imagination displayed by academic staff, whose education and training one would think encouraged imaginative and radical thinking, remains one of the paradoxes of university life. Comfortably tenured academics may not feel that the incentive to divert their attention towards these managerial concerns is particularly overwhelming. However, those who are interested in maintaining or encouraging the flourishing of research and higher education might do well to ensure that serious attention is paid to such matters before expecting largesse to be poured upon them.

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Are we grown up?

SIR — As always when the issue of extraterrestrial intelligence is raised in your columns (*Nature* 355, 581; 1992), it is implicitly assumed that 'they' are as eager to contact 'us' as 'we' are to contact 'them'. In other words, mankind (at least the (small?) part of it advocating the SETI projects) considers itself sufficiently grown up and evolved to apply for galactic community membership (supposing the latter exists). Are we?

Supposing that extraterrestrial intelligence exists, it is probably intelligent enough to hide itself from us, at least for the time being. Understanding that their superior technology would probably cause mankind to be even more destruc-

tive of itself and its planet (or, if not, to bring about unbearable suffering to the countless number that already suffer), galactic community members would refuse to take the responsibility of manifesting themselves.

On this view, managing to bring about equal life standards for all of mankind and to halt environmental disaster would be a far more effective means in the search for extraterrestrial intelligence than merely listening or sending messages to the voids.

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Insecticide death

SIR — It is true that aluminium phosphide (ALP) is being misused in northern India (see *Nature* 353, 377; 1991) for both suicide and homicide. ALP tablets look like a medicinal preparation, and the victims are often ignorant rural brides. But K. S. Jayaraman's statistics for "all pesticide poisoning" fall far short of the reality. The numbers of deaths cited, 192 in 1988–89 and 664 in 1989–90, represent a very small fraction of the toll from insecticide poisoning in India. In the state of Maharashtra alone, which represents about 9 per cent of the population of India, the figures for detected pesticide poisoning in the most recent three years for which records exist are shown in the table.

	1990	1989	1988
Organophosphate compounds	1,387	1,208	1,061
Organochloro compounds	1,239	1,105	786
Carbamate	424	390	308
Total	3,050	2,703	2,155

Common pesticides misused in 1990 (more than 50 fatal suicide/homicide cases) are: Organochloro compounds: endosulfan (1,029); organophosphate compounds: dimethoate (309), Thimet (183), monocrotophos (179), fenitrothion (153), Dimecron (125), Nuvan (100), malathion (98), fenitrothion (74), methyl parathion (67), Metafastox (51); carbamates: Baygon (406).

Some of these insecticides have very low LD₅₀ values, such as approximately 1 mg/kg for thimet (phorate), others in the range 15–250 mg/kg; barring malathion which by itself is comparatively less toxic. ALP is not yet a problem in Western India.

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Japanese success

SIR — One reason not advanced by Alexander Kennaway (*Nature* 355, 198; 1992) for the success of the Japanese science and technology effort is that, in Japan, science and technology have fully proved their benefits to the public, in stark contrast to the situation in the Western world. This is the result of several decades of outstanding applied research and development by Japanese companies. The Japanese government then encouraged companies to undertake more and more basic research, the potential commercial results from which were quickly and efficiently realized. By this process, the Japanese 'person in the street' received a clear demonstration of the benefits of basic research. Only then did Japanese universities begin to receive research grants in similar amounts to those with which the Western world has been familiar for some time. It seems unlikely that Western governments will release more funds for university research until the average citizen is convinced that the expenditure of his/her tax dollars will provide more personal benefits than is now the case.

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Trying times for human insulin

Simon P. Wolff

The introduction of human insulin to treat diabetics seemed straightforward. What can account for the problems that have followed?

THERE is no such thing as a safe drug. There are surprising allergic responses, interactions between drugs, peculiar responses resulting from the vicissitudes of individual metabolism or unusual diets, severe reactions caused by accidental or intentional overdose and adverse effects linked to other disease. Drugs with overt and easily discernible toxicity are easily screened out during basic toxicity testing, during volunteer and clinical trials and, finally, during follow-up. There are few disasters, simply because pharmaceutical companies have a natural interest in not poisoning their clients. If adverse effects crop up during post-marketing surveillance then these are carefully scrutinized and identifiable risk factors are searched for. If the adverse effects are serious and strike at random then the drug is rapidly withdrawn. If they are less serious, and risk factors are easily identifiable, clear warnings are added to the packaging and sale continues.

But there will always be a fraction of the patient population which imagines unpleasant symptoms with a new drug, or with a new formulation of a drug, and it is inappropriate to consider withdrawing the drug until the response of a large section of the drug-taking population is known. It would be wrong for one small, vociferous group to be the direct cause of withdrawal of a drug that might benefit the vast majority of sufferers. Pharmaceutical companies and regulatory authorities thus often have to cooperate in the face of front-page banner headlines concerning the complaints of a few individuals. The continuing controversy over human insulin is an illustrative example.

The innovation

The development of human insulin (particularly using recombinant DNA technology) seemed an attractive and logical idea. There were doubts that it would solve the long-term problems of the individual with diabetes, and few illusions that it would offer a clinical advantage over the well-established treatment using animal insulin, but no problems were anticipated concerning switching patients from the animal forms to the human¹. Gradually, however, this optimism began to be eroded with the first reports of 'hypoglycaemia unawareness': the loss of sensation in the diabetic person that his or her blood glucose

levels were dropping dangerously low.

In the nondiabetic individual, blood glucose levels are tightly controlled by the counter-regulatory effects of insulin-dependent storage of excess glucose (as insoluble glycogen in the liver and muscle) and adrenalin stimulation of glycogenolysis and gluconeogenesis. Too much glucose, and it is removed from the circulation; too little, and it is generated from stores or by catabolism of fatty acids and amino acids. The brain is critically dependent on glucose: when levels are too low adrenalin is released, causing various adrenergic (more properly, autonomic) symptoms such as sweating and palpitations related to the flood of catecholamines. In addition, there are central nervous system (neuroglycopenic) symptoms such as aggressiveness, confusion, lack of concentration and visual disturbances. The individual with diabetes is therefore warned of a hypoglycaemic event by several characteristic symptoms and can avert a severe attack by ingesting a few grams of glucose. If the individual does not respond to the warning signs in time, or loses awareness of the attack, there is a risk of coma or even death.

Teuscher and Berger were the first to cast doubts on human insulin when they suggested² that individuals in Switzerland who had changed from porcine to human insulin experienced symptoms that could have resulted in a delayed or diminished response to a hypoglycaemic attack. They cited three case histories and the results from interviews of 66 patients who, out of a total of 176, had reported a change in their hypoglycaemia symptoms from autonomic to neuroglycopenic. Criticism of Teuscher and Berger's paper was robust. A switch in symptoms was said to be "biochemically counter-intuitive"; the study was accused of being designed to reach predetermined conclusions and did not "warrant statistical analysis"³. Undeterred, the same group published⁴ a double-blind randomized crossover trial of human and porcine insulin which seemed to confirm the initial observations. This paper was criticized on the basis of biased patient selection and misallocation of autonomic and neuroglycopenic symptoms — for example, raised heart beat and palpitations may well be autonomic, but what about 'anxiety'?^{5,6}. But an editorial in the *Brit-*

*ish Medical Journal*⁷ pointed out that there had been two reports of unexpected and severe hypoglycaemia after transfer to human insulin among British patients^{8,9}, and that the British Diabetic Association had received many unsolicited letters from patients noting a problem in themselves. Reports in the popular press began to appear concerning an increasing frequency of unexpected deaths in young diabetic patients. A London pathologist considered it unusual that he had examined 19 young diabetics who had died suddenly over an 18-month period. Headlines such as: "Insulin warning after doctor dies" (*The Guardian* 1 August 1989) and "Medical alert after mystery diabetic deaths" (*Daily Telegraph* 22 August 1989) implied a link between the introduction of human insulin and severe adverse effects.

Tattersall and Gill have now summarized the difficulties in making a systematic analysis of such rare deaths, and in allocating them to a particular cause¹⁰. They examined 50 cases of unexpected death in diabetics below the age of 50. After exclusion of those with some clear cause, 30 deaths could not be explained. Twenty-two of these individuals were young (mean age 30), did not have complications of the disease and had gone to sleep apparently well but were found dead the next day. Given that a certain number of young diabetics die unexpectedly each year, even before human insulin became widely used, Tattersall and Gill concluded that it is impossible to say whether the 22 deaths in one year represents a real increase. Indeed, it is possible that the pathologist's observation mentioned above resulted from a change in government practice from using many clinical pathologists to using just a few.

A physiological mechanism?

A physiological explanation for a distinct effect of human insulin was offered by a Dutch group, which reported that healthy individuals infused with human and porcine insulin seemed to have a smaller increase in measured autonomic responses when hypoglycaemia was induced by human insulin than with porcine insulin¹¹. The authors tentatively ascribed this observation to a greater penetration of the blood-brain barrier by the slightly more lipophilic porcine

insulin, which could lead to differential direct effects on the central nervous system. They noted no differences in neuroglycopenic symptoms. The study was criticized for its lack of relevance to diabetes (the subjects did not suffer from the condition) and for its lack of methodological detail concerning description and assessment of hypoglycaemia symptoms, which are hard to quantify^{12,13}. Criticisms of these and other studies¹⁴⁻¹⁹ are typical of those made of observations of small effects: the wrong end-point is being measured; patients are asked misleading or leading questions; the research is not properly randomized or blinded. So far, there is no consensus on the physiological basis of a slight (but clinically important) difference in physiological response to human insulin, which in any case remains unproven, and nobody seems able to agree on ways of measuring any difference. Indeed, academics who are sceptical of the case against human insulin have been accused of being little more than in the pay of drug manufacturers.

In many scientific disputes, editors of mainstream publications eventually allow those who agree to disagree to slug it out in the minor literature, where things are quietly forgotten. But this dispute concerns real or perceived injury to many individuals who require a life-saving drug on a daily basis. Television programmes have featured articulate and concerned individuals with diabetes presenting their symptoms as caused without doubt by human insulin. Into this heady mix of personal acrimony and bad-tempered academic dispute have now stepped the lawyers.

A consortium of lawyers in the United Kingdom is currently coordinating claims made on behalf of diabetic individuals who believe they have suffered adversely. In principle the lawsuit might eventually involve 20,000 of the country's 250,000 diabetics (the proportion that has reported the ill effects) resulting in potential claims of many millions of pounds. Press publicity has enormously increased the numbers who feel injured and litigious. But who should be sued, for how much, and on what grounds? The manufacturers can justifiably claim that they carried out all relevant tests. The regulators were satisfied. All subsequent scrutiny of animal and human insulins suggest that any differences must be so vanishingly small that they are likely to become apparent only in a very large randomized controlled trial of unprecedentedly complex design. There is no evidence of skulduggery: altered or incomplete notebooks are conspicuous by their absence.

Some fault does, however, seem to exist. Why was there such a rush to switch patients from animal to human

insulins, and why was the campaign so effective? The drug was introduced in the United Kingdom in 1982. It had only 5 per cent of the market three years after its introduction, but seems suddenly to have expanded so that by 1989 it was used by most individuals with diabetes, who generally had no complaint. With the benefit of hindsight, it might have been wiser to avoid this surge to market dominance.

Physicians were probably persuaded to change their patients' prescriptions so rapidly for three reasons. First, promotional literature seemed to claim that human insulin was likely to be far less immunogenic than animal insulin. But this was not true, at least for porcine insulin, although there may have been problems with bovine insulin²⁰. Second, advertising campaigns made the new insulin sound far superior and thus encouraged patients to ask for it. Third, physicians and pharmacists mistakenly believed that animal insulins were being discontinued.

On what basis will any legal case come to court? The clinical evidence is unconvincing — there is little agreement about the reality of the phenomenon nor of its physiological mechanism. Any real cases of human insulin-related hypoglycaemia unawareness are likely to be buried beneath a morass of 'me-too' complainants. Nor is there firm evidence to suggest that any deaths or serious injuries have been caused by hypoglycaemia peculiar to human insulin. Nor is there a possibility of 'no fault' compensation because 'fault' has yet to be established. It is also notable that there is no equivalent controversy over human insulin in the United States, perhaps because warning notices of possible altered hypoglycaemia awareness were introduced on packaging earlier. Certainly, there seem to be remarkable national differences. In Switzerland, there was a reported doubling of hospital admissions for severe hypoglycaemia as the market share of human insulin climbed from 5 to 60 per cent over a four-year period. By contrast, hospital admissions in Holland decreased slightly even though there was a similar rise in market share over the same period²¹.

The human insulin story will give pharmaceutical manufacturers and regulators pause for thought. Manufacturers have introduced a new drug in good faith with no prior evidence of adverse effect and with no subsequent firm evidence of any such reported effect. But they now find that a profitable product has been tarnished and runs the risk of further opprobrium if or when a case comes to court. Manufacturers may in future have to think carefully about bringing new drugs to the market place unless they are anticipated to have clinical advantage,

which human insulin does not. Regulators may have to consider tempering enthusiastic introduction of a new drug so that sufficient time can pass for any adverse effect, no matter how unlikely, to become apparent. They may therefore have to regulate and monitor drug marketing as well as safety and efficacy. A preliminary period of strict control of prescriptions is not part of the UK Medicines Act of 1968, but is needed.

Finally, is there a lesson for the clinical scientists that can be applied to the patients in their care? One has the impression from certain sections of the clinical community that the whole debate has been a sorry waste of time. As one recent editorial ran: "Even journalists and media commentators have been led to ask whether some of the heavily criticized contributions in the field, and in particular the self-evidently unscientific study which began the controversy, should have been published in reputable medical journals at all"²². Strong stuff for what was an interesting and publishable preliminary study. Even if the original suggestion eventually turns out to be wrong, much useful information will have been gathered on the way. Everyone is now undoubtedly much more aware of the issues of sudden death in diabetes, severe hypoglycaemia, and risk factors such as possibly unnecessarily strict control of blood glucose²³. Cool and careful appraisal of the problem will undoubtedly help diabetics live more comfortable and confident lives. □

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Eat your broccoli (and brussels sprouts)

Masses of circumstantial data link diet and disease, including a new report about a chemical in broccoli that detoxifies carcinogens *in vitro*, but it remains the case that very little is really known about food as medicine

THE idea that health is related directly to diet is an appealing one. If you just eat right dread disease can be kept at bay. Particularly in the United States where, during the past decade both the federal government and private scientific organizations such as the National Academy of Sciences have become powerful advocates of a 'healthy diet', eating right is associated with 'take charge' type of people who are in control of their lives, and therefore their bodies.

Take charge people eat oat bran, and lots of cruciform vegetables — like broccoli. They do not get cancer.

Studies too numerous to count, published in journals refereed and not, point to a link between diet and various forms of disease. The high-fat diets characteristic of Western nations are blamed for heart disease and cancer that, in incidence, is greater than that among peoples whose diets are rich in grains and vegetables. The official word from the Food and Nutrition Board of the Academy's Institute of Medicine is this: eat five or more servings of vegetables and fruits daily, especially green and yellow vegetables and citrus fruits. The advice is repeated on television by spokesmen for grocery store chains.

Fibre is important too. Last year, oat bran was 'in'. Now, thanks to a recent report in the March *Proceedings of the National Academy of Sciences* (89, 2394–2403), broccoli is in vogue. Broccoli contains a chemical called sulforaphane, first synthesized in 1948, that appears to be a potent inducer of an enzyme that detoxifies carcinogens in mouse hepatoma cells.

Paul Talalay and his colleagues at The Johns Hopkins University School of Medicine reported in *PNAS* the development of a test for the 'rapid detection of inducers of enzymes that protect against carcinogens.' Specifically, they measure an agent's capacity to stimulate quinone reductase in murine hepatoma cells grown in microlitre plate wells, and began with an analysis of various components of broccoli because of the many epidemiological studies that suggest that people who eat broccoli tend not to get cancer. Talalay says he chose it also because broccoli is widely consumed in the United States, despite George Bush's famous declaration that now that he is President he does not have to eat his broccoli anymore.

Talalay's rapid assay was developed with several premises in mind. First, that 'dietary composition is a major determinant of cancer risk in humans and experi-

mental animals'. Second, that the 'consumption of vegetables, especially crucifers, reduces the risk of developing cancer'. And, third, that malignancy is regulated by so-called phase I enzymes that activate carcinogens and by phase II enzymes that detoxify them.

Focusing attention on chemicals in food that appear to detoxify carcinogens (rather than on carcinogens or mutagens that have been well-studied), Talalay and his colleagues report that their test turned up a number of anticancer substances, of which sulforaphane is the most potent. Peppers, potatoes and tomatoes appear to be low inducers of anticancer activity, whereas some vegetables (red leaf lettuce, beets, bok choy and cauliflower among them) actually showed some capacity for cytotoxicity. 'Cytotoxicity measurements are important,' Talalay writes, 'because phase II enzyme inducers may be toxic and/or carcinogenic.'

What are we to make of all this? Not much if you are thinking about dinner. The Talalay data, if they hold up in further experiments, represent a potentially useful step in the direction of sorting out the connection between food and disease at the level of cellular mechanism. A test for naturally occurring agents that may exert a protective effect could be useful in the long run in the development of medically useful drugs. In any case, efforts to distinguish fact from wishful thinking in the diet and cancer business are commendable.

But, contrary to enthusiastic press reports, it is premature (to put it mildly) to suggest that the broccoli-haters of the world have a duty to consume the stuff in the name of a long and healthy life. In fact, as others have noted before, taking all the data about health and disease and adding them together, still does not make the case that the average person (with no known genetic predisposition and no present disease) can prevent cancer by eating 'right'.

This is not an endorsement of a diet of greasy hamburgers and cotton candy. Rather, it is simply another occasion to consider the complex issues of doing science, estimating risk, and then communicating that risk to the public in an arena in which people are able to make individual choices (as opposed, for instance, to such global cases of potential risk as Chernobyl or global warming).

Even a brief review of past cases shows the risks of simplifying risk prevention. Beta carotene, a precursor of vitamin A, is

thought to be an anticancer compound on the basis of data from dietary questionnaires and from some animal studies. In a clinical trial of 1,805 patients with non-melanoma skin cancer, however, beta carotene failed to reduce the incidence of new cancers (*The New England Journal of Medicine* 323, 789–795; 1990). An article in the same issue of *NEJM* (795–801) reported that clinical trials of 13-*cis*-retinoic acid failed to prevent recurrences of original tumours of the head and neck but did appear to prevent the development of new or 'second primary' tumours. Tests did not confirm expectations.

Then there is the unhelpful biological complication that Talalay himself referred to: some agents that induce anticancer enzymes may also be toxic. This phenomenon of the double-edged sword is a frequent impediment to clean data in cancer metabolism and pharmacology.

Animal biology presents another reason for caution. Despite diligent efforts of hundreds of scientists, it is not yet entirely clear why an agent that is carcinogenic in one animal is safe in another. Penicillin injected into the veins of a rat causes sarcoma. Alcohol is a carcinogen in humans but not in rodents. Crystalline silica causes lung tumours in rats but not in mice. Most people who get lung cancer smoke, but most people who smoke do not get lung cancer.

In short, when it comes to nutrition and disease, ignorance exceeds knowledge and a little knowledge, while scientifically valuable, cannot be readily translated into a menu for health. What is needed? The answer is all too predictable. Not only more, but better, research in nutrition. For fifty years, the science of nutrition was the elucidation of nutrients and deficiency states — vitamin D and scurvy, for instance. There followed important decades of epidemiological study during which associations about diet and disease accumulated. Now the era of molecular nutrition should be in full flower but it is not here quite yet. The field deserves the attention of the most sophisticated researchers.

Every month or so another 'broccoli' story makes news, reflecting an understandable desire for a silver bullet, a single answer. But given the present level of knowledge, there is only one right answer about diet and health. Do what your mother told you to do — eat a balanced diet and get some exercise. And hope you inherited good genes.

Barbara J. Culliton

Worlds of difference

John Mollon

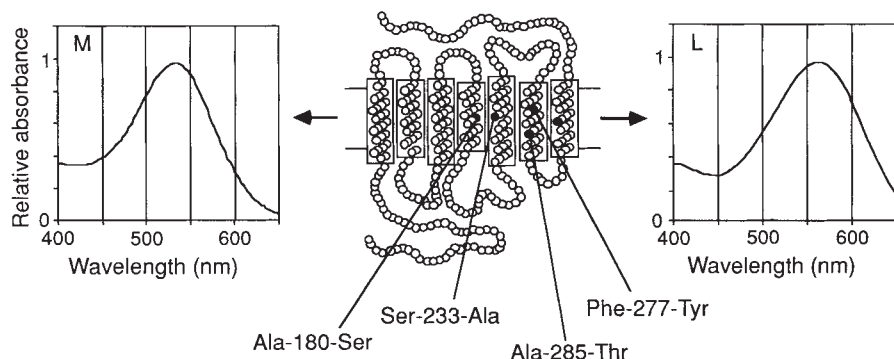
YOU and I may pass through our lives in different perceptual worlds. Although we both may enjoy colour vision that is nominally normal, coloured objects that look alike to you may look distinctly different to me, and those that look different to you may look identical to me. And this small but irreducible discrepancy in our sensations can be traced to a single nucleotide difference in our X chromosomes. That is the implication of two papers published on pages 433¹ and 431² of this issue.

It was in these columns in 1881 that John William Strutt, second Baron Rayleigh, introduced the colour match that has ever since served to classify normal and abnormal vision: the observer is asked to find the ratio of red to green light that matches a monochromatic orange³. More recently, *Nature* has twice (and independently) carried reports that the distribution of such 'Rayleigh matches' in a colour-normal population is bimodal^{4,5}. And those two papers may have been anticipated by an earlier study of over 2,500 Yugoslavs, where a provocative bimodality was reported without comment⁶. Certainly everyone who has measured Rayleigh matches — including Lord Rayleigh — agrees that normal observers differ in their responses (see for example refs 7–9). But what is the physiological basis for these variations in perception?

Our daytime vision depends on light-sensitive molecules embedded in the concertina-like membranes of the cone cells of the retina¹⁰. Each of these photopigment molecules consists of a large protein that has been bound to retinal, a derivative of vitamin A. The protein component has the 'heptahelical' structure characteristic of all G protein-coupled receptor molecules (see my previous News and Views article¹¹). Small variations in the amino-acid sequence of the protein (or 'opsin') yield pigments with peak sensitivity in different parts of the spectrum.

There are usually taken to be three normal human cone pigments, with peak sensitivities in the violet, the green and the yellow-green regions of the spectrum. But in 1983 a microspectrophotometric study of retinæ from human patients suggested that there were two alternative types of long-wave cone, which were associated with different psychophysical sensitivities to red light¹². And when, in 1986, Jeremy Nathans and his colleagues first published nucleotide sequences for the opsin genes, they noted that the normal long-wave gene was polymorphic: slightly different

amino-acid sequences could be inferred for the corresponding pigment according to whether the gene was derived from Nathans' own genomic DNA or from a complementary DNA library synthesized from messenger RNA from eyes obtained at autopsy¹³. One of those polymorphic sites corresponded to number 180 in the amino-acid sequence. More recently, phenotypic and genotypic correlations in New World monkeys have strongly indicated that this site is one of those that control the wavelength



The visual photopigments are members of the class of heptahelical receptors, each consisting of a palisade of seven membrane-spanning helices. In this figure, the filled circle in the seventh helix indicates the lysine residue at which the molecule is bound to 11-*cis*-retinal, the chromophore. The other filled circles indicate four amino-acid sites that probably control the spectral tuning of the long- and middle-wave cone pigments. For the four sites, the alternative amino acids are shown at the bottom of the figure: in each case, the amino acid to the left is associated with a pigment shifted to middle wavelengths and the amino acid to the right is associated with a red-shifted pigment. The graph on the left shows the absorbance spectrum of a normal human middle-wave cone, that on the right the spectrum of a normal long-wave cone. Absorbance data are from ref. 12.

at which the pigment has its peak sensitivity^{14,15}.

So far, however, in judging which DNA sequence gives a photopigment of a given spectral sensitivity, we have been restricted to the indirect evidence from correlating genotypes with the phenotypes of monkeys or of colour-normal and colour-deficient men. Now two groups have used tissue culture to express genes for the opsins of the three normal human cones. Oprian *et al.*¹⁶ started with chemically synthesized artificial genes, whereas Merbs and Nathans¹ (page 433 of this issue) began with cDNA clones isolated from human retinæ. In both cases, when the gene products were combined with retinal and were purified, they proved to have absorbance spectra resembling those recorded earlier from human cones by microspectrophotometry and electrophysiology^{12,18}. An added twist of Merbs and Nathans' work is that they have reconstructed two versions of the long-wave pigment, differing at site 180. As suspected, the two gene products have different absorbance spectra: the pig-

ment with alanine at site 180 exhibits a peak sensitivity at 552.4 nm, whereas that with serine at site 180 has a peak at 556.7 nm.

This result is prettily complemented by the second paper² in this issue (page 431). Winderickx and colleagues, in Seattle, have measured the Rayleigh matches of 50 colour-normal males and have analysed DNA from each subject. Using the polymerase chain reaction (PCR) to amplify the relevant part of the long-wave gene, they find that 62 per cent of subjects exhibit the code for serine at site 180 and 38 per cent the code for alanine. As would be predicted from the primate work and from the new absorbance curves of Merbs and

Nathans, the subjects with serine at 180 exhibit a higher sensitivity to red light. Qualitatively similar results were described by Neitz *et al.* at a meeting in January¹⁷, although those authors used PCR primers that concurrently amplified the corresponding parts of long- and middle-wave genes: they report a strong, but more graded relationship between the Rayleigh match and the ratio of the two alternative nucleotides that determine whether alanine or serine occurs at site 180.

The significance of these discoveries for psychologists cannot be exaggerated. Here is a case where a difference of a single nucleotide places people in distinct phenomenal worlds and where we know almost all the steps in the causal chain from gene to molecule to neural signals; only the final steps from cortical activity to sensation elude us. It is the first such case in psychology. It cannot be the last.

This is not to say that the genetics of colour vision are all tied up. Here are four examples of unsettled issues.

First, Neitz *et al.*¹⁴ last year put for-

ward an attractively simple theory of the spectral tuning of cone pigments, suggesting that the difference between the human long- and middle-wave pigments depended on three amino-acid substitutions only (at sites 180, 277 and 285) and that these substitutions were additive in their effects on the wavelength of peak sensitivity. A British group (which includes myself) has questioned this theory, proposing that at least one further site (233) is involved, possibly in a nonadditive fashion^{15,18}. The evidence is that all ten primate species so far investigated exhibit a non-hydroxyl-bearing residue at site 233 of the long-wave (> 560 nm) pigment, whereas, in all eight cases where the species exhibits a pigment peaking below 540 nm, that pigment has a hydroxyl-bearing residue at site 233. So it is instructive that Winderickx *et al.*² have identified two atypical subjects who had hydroxyl-bearing amino acids at site 233 of their long-wave pigment: both were less red-sensitive than would be expected from the amino acid they exhibit at site 180.

Second, the Seattle group maintains, as Nathans and colleagues have always done, that an individual male has only one long-wave gene (though perhaps several middle-wave genes)². Neitz and colleagues, on the other hand, hold that more than one long-wave gene may be present, and expressed¹⁷: so many men are 'pseudo-heterozygotes' for the Ser/Ala substitution at site 180. Their reasoning is that the spread in Rayleigh matches is less than primate data would predict if the variance came mainly from a site-180 polymorphism in a single long-wave pigment. Two alternative explanations would be that the Ala-180 version

of the pigment has a higher effective optical density, and thus its relative sensitivity in the red is enhanced, or that the magnitude of the spectral shift due to site 180 depends on other, concurrent, substitutions.

Third, Mathew Alpern has for 15 years held that anomalous trichromats achieve their residual red-green discrimination through the presence of either two versions of the normal long-wave pigment or two versions of the middle-wave pigment⁷. Does his hypothesis draw support from the normal polymorphism now demonstrated?

Finally, given the Ser/Ala polymorphism in the male population, there ought to be women who are heterozygous for

this substitution and carry different alleles on their two X chromosomes. Because of random X-chromosome inactivation, only one of the two long-wave pigments will be expressed in any individual cone. In female platyrrhine monkeys, the two corresponding types of cone are able to sustain a colour-opponent signal²⁰. Does this mean that a heterozygous woman can be tetrachromatic, experiencing an extra dimension of hue that must forever be forbidden to her male conspecifics? □

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DNA TRANSCRIPTION

Zinc standard for economy

Ben Luisi

THE hypothesis that zinc-mediated peptide loops can interact specifically with nucleic acids¹ has rapidly turned into a general principle, as ever-more distinctive examples of such folds are identified. Amino-acid sequence comparisons have indicated that there are three main structural classes of zinc-bearing module. Two have been characterized, both of them in complex with target DNA, by crystallographic studies of two vertebrate transcription factors; one is Zif268 (ref. 2), which represents the zinc-finger class originally found in the transcription factor TFIIIA (ref. 1), and the other is the glucocorticoid receptor DNA-binding domain, representative of the zinc domain of steroid/nuclear receptors³.

A third zinc-mediated fold now makes its bow in reports on pages 408, 448 and 450 of this issue⁴⁻⁶. The authors describe the NMR structure^{5,6} of the DNA-binding domain from the yeast transcription factor, GAL4, and the crystal structure of the protein/DNA complex⁴. These studies show that GAL4 has a strikingly distinctive metal coordination scheme and mode of DNA binding.

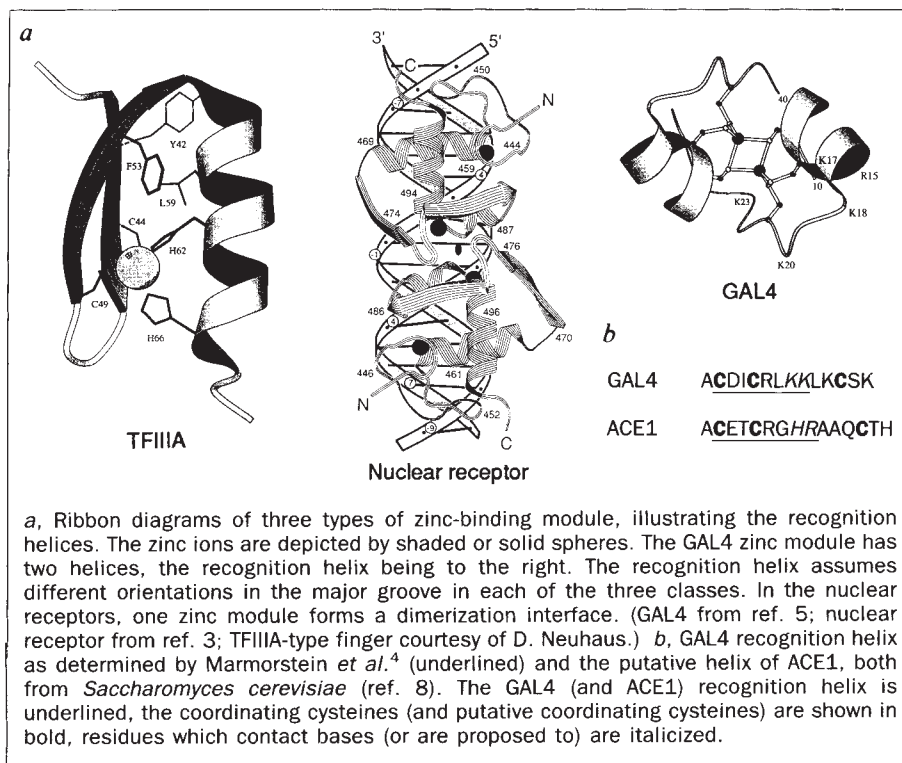
The salient feature of the GAL4 DNA-binding domain, and one which distinguishes it from the two other zinc-bearing domains, is its metal cluster. In a coordination scheme reminiscent of that of the metallothioneins, GAL4 binds two Zn(II) ions through six cysteines such that the metals share two of the ligands. In the two other structurally characterized modules, the Zn(II) ions are individually coordinated by four ligands. In the zinc-finger motif, the metal is coordinated by two histidines and two cysteines to yield a module of roughly 30 amino acids (about the same size as the GAL4 zinc module); and in the nuclear-receptor zinc modules, two

metals are each bound by four cysteines to form a compact domain of 70 amino acids.

Despite their structural differences, the three classes of zinc-binding motif have a common role in exposing an α helix which lies in the major groove of the DNA and directs interactions with the bases of the target sequence (*a* in the figure). Zinc coordination caps either the amino or the carboxy termini of these recognition helices. Alpha-helices are also found as recognition elements in the helix-turn-helix motif of prokaryotic genetic regulatory proteins and in the homeobox of eukaryotic proteins. Here, the recognition helix is supported by a core of hydrophobic residues which forms a structural brace. In the zinc modules, metal coordination, supplemented by hydrophobic interactions, carries out the corresponding architectural function with structural economy.

The three structural classes exhibit three distinct modes of DNA binding. For example, proteins with zinc fingers are often composed of a concatenation of fingers, and, as seen in the Zif268/DNA complex, each can in principle recognize a sequence triplet². Consequently, these proteins can interact with elements that are composed of contiguous, single-finger target sites by wrapping around the DNA so as to follow the major groove continuously. Glucocorticoid and certain related steroid/nuclear receptors form homodimers and bind palindromic targets on one surface of the DNA, much like the prokaryotic and phage regulatory proteins³. The DNA targets of GAL4 are also palindromic, and the GAL4 DNA-binding domain dimerizes symmetrically on the DNA⁴. The domain

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contacts target sites that are one-and-one-half helical turns apart and therefore lie on opposite surfaces of the DNA. The dimerization elements meet at the dyad axis of the complex, above the minor groove. The linker between the dimerization interface and the zinc-module tracks along the phosphate backbone through contacts made by a string of basic residues.

Eukaryotic transcription factors often collaborate in regulating gene expression by forming specific assemblies on target DNA elements. The GAL4/DNA complex provides clues as to how such collaboration could be achieved in particular cases. The mode of binding leaves an exposed major groove at the centre of the GAL4 binding site, and, as Marmorstein *et al.*⁴ speculate, room for the binding of further transcription factors.

Dimerization of the GAL4 DNA-binding domain occurs through the intermeshing of small, hydrophobic residues emanating from two parallel, coiled-coil helices⁴. This dimerization interface is similar to that in the 'zipper' transcription regulatory proteins, where the interface is formed by hydrophobic interactions of leucines. GAL4 also has a DNA-independent dimerization segment, but it has not yet been structurally characterized. In the absence of DNA, the GAL4 DNA-binding domain is monomeric, and the NMR observations imply that the dimerization domain is disordered⁶. Could DNA binding nucleate the folding of the dimerization domain, resulting in cooperative association of two monomers? There is an example of such a phenomenon, and it

comes, interestingly enough, from a zinc module. The dimerization interfaces of the DNA-binding domains from the oestrogen and glucocorticoid receptors seem to be disordered in solution⁷, but the interface is well ordered in the DNA complex of the latter³. Both of these proteins are monomeric and bind their targets cooperatively. Whether such an effect also occurs in a full-length transcription factor remains to be seen.

Like the other zinc-module classes, GAL4 has a number of highly homologous relatives which undoubtedly fold with similar coordination schemes and recognize related DNA targets through variations in a small number of critical amino-acid/base contacts in the half-sites. In the nuclear receptors, discrimination of closely related target sequences is achieved in part through a few critical residues in the zinc-module domain and also through dimerization, which dictates the spacing between half-sites and may affect the preferences of subgroups of receptors for inverted rather than direct repeats³. Analogously, the linker connecting the zinc-recognition module and the dimerization domain affects the separation of the GAL4 monomers on the target site and, consequently, the preference for half-site spacings. The GAL4 family members may discriminate between targets with varying spacing between sites by using different linker lengths⁴.

The GAL4 structure may provide some insight into the transcriptional activation mechanism of ACE1, another yeast metal-bearing transcription factor to which it could be distantly related.

Upon ligating Cu(I), ACE1 can bind specific DNA targets to regulate the expression of metallothionein genes⁸. It has been proposed that ACE1 coordinates metal in metallothionein-type clusters, and it has been shown that the DNA-binding function coincides with the metallothionein-like domain. In this domain, eight Cu(I) ions could be coordinated by twelve cysteines in a cubic arrangement. Intriguingly, a segment of ACE1 within this domain is similar in sequence to the recognition α helix of GAL4. This segment may form an exposed helix that is part of the ACE1 DNA-binding surface (b in the figure).

Possibly, along with zinc modules comes the requirement to maintain a precise zinc homeostasis that presents the metal at the right place and time. Such homeostasis would require high fidelity, because it would directly affect the machinery of gene regulation. ACE1 shows how metals can directly affect gene expression; zinc homeostasis might likewise be involved in gene regulation through its effects on the zinc-bearing transcription factors. It is curious that in those organisms most noted for genomic economy — the prokaryotes — no zinc-bearing transcription factors have yet been identified (although they certainly have genetic regulatory proteins that bind other metals and mediate detoxification responses). Could the prokaryotes have avoided the 'hidden costs' of the regulation required for maintaining zinc modules?

The distinctive folds of the GAL4, TFIIIA and steroid/nuclear receptor zinc modules give the impression that zinc may have been discovered several times in the eukaryotes as a versatile scaffold for transcription factors. Zinc is also used in proteins from retroviruses and bacteriophages which are proposed to recognize single-stranded nucleic acid and which form a distinct structural fold⁹ from the three described here. There remain many uncharacterized structures supported by zinc in other eukaryotic regulatory proteins¹⁰. They too may well have distinctive folds, and may give us further insight into the mechanics of gene regulation. □

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Reversal ideas up-ended

Phil McFadden

RECENT suggestions that virtual geomagnetic poles have a strong tendency to follow one of two paths during a geomagnetic reversal are called into question on page 400 of this issue¹, where Valet *et al.* suggest that the available data have been overinterpreted. In doing so, the authors upset an attractive hypothesis that links motions in the Earth's core with convection in the mantle and thereby with plate tectonics.

The polarity of the Earth's magnetic field remains constant for long intervals of time (ranging, in a random manner, from hundreds of thousands to millions of years) and then, for reasons we do not yet understand, will suddenly reverse. What happens in the 10,000 or so years it takes the field to reverse is both a puzzle and an important element in elucidating the reversal mechanism. The suggestion made last year²⁻⁵ was that, at least for the last few geomagnetic reversals, the field retained a simple geometry during reversal and that the virtual geomagnetic poles simply swung from North to South, or vice versa, almost always along a path through North and South America or else (less frequently) through eastern Asia and Australia, 180° away.

However, the Earth's core (where the geomagnetic field is generated) cannot, by itself, 'know' its orientation in space, and so cannot have any memory from reversal to reversal of geographical location. In contrast, the time constants of mantle convection are orders of magnitude longer than those of the core, and the implication is that the mantle, probably through the temperature distribution at its base, is having an observable effect on the behaviour of the core. This has profound implications for a causal link between core motions and plate tectonics, and the suggestion was greeted with much excitement. The evidence presented was visually compelling, but Valet *et al.* now challenge the interpretation, claiming that the data are not inconsistent with a uniform random choice of transitional path.

Mapping

To map the configuration of the field at any time it is necessary to have synchronous observations from sites that are geographically well distributed. To map the changing configuration during a reversal one needs a sequence of such observations well distributed in time. As it happens, the remanent magnetization of rocks provides us with a record of the geomagnetic field. Unfortunately though, the palaeomagnetic recording

process has been sporadic, often distorted, and susceptible to subsequent alteration. Thus the individual observations are difficult to read correctly, they are poorly distributed in space, it is difficult to find a record that captures a reversal transition, and there is currently no independent way to assign a time-scale within a transition with sufficient precision and accuracy to align observations from different sites. Consequently, it is not possible to construct the maps we desire. The challenge then is to distil from the observations any systematics that may help us to understand the processes governing the behaviour of the core.

Given the direction of the magnetic field at some point on the Earth's surface it is a simple matter, assuming that the geomagnetic field is a dipole, to calculate the position of the north pole. The position of this hypothetical pole is then referred to as a virtual geomagnetic pole (VGP). If the geomagnetic field at the Earth's surface is largely dipolar (as it is now, and as it appears to have been throughout most of the past) then VGPs from different points on the Earth will coincide. Small variations in the position will represent noise due to the non-dipolar content of the field. The VGP has therefore been a physically meaningful and powerful tool for tectonic palaeomagnetism. If the field retained a principally dipolar configuration during a reversal, then the VGPs from different sites would follow similar paths. But, without some external control, one would expect the path to differ from reversal to reversal.

If, however, the field has a complicated, nondipolar geometry, the VGP degenerates into a physically meaningless transformation and VGPs from different points will be widely dispersed. Transitional VGP paths from different sites will then disagree. Until last year, it was generally accepted that the transitional field was strongly non-dipolar⁶ and so had a complicated geometry.

In the recent data, the individual VGP transition paths appear to be reasonably well confined to a longitudinal sector for much of each reversal. If, within a given reversal, there were a common preferred path for all sampling sites, then this would suggest a simple rotation of a largely dipolar field. However, the existence of a second preferred path, while still requiring a relatively simple geometry for the transitional field, rules out such dipole dominance⁴. There are in fact many relatively simple geometries that could

produce antipodal paths, and Clement⁴ has identified one with a dominant octupolar field.

The truly exciting suggestion, however, is that several reversals share the same preferred path, for this would indicate that the mantle has some direct control over the affairs of the core. Tric *et al.*², in a presentation that certainly suggests a preferred path, represented 48 transition records as a rose diagram, showing the number of reversal transition paths as a function of the mean longitudes of those paths. Laj *et al.*³ plotted all the individual VGP positions (see the cover of *Nature*, 6 June 1991) in a presentation that almost compels one to accept the hypothesis of a preferred path. However, this latter presentation is naturally biased towards those paths with a large number of VGPs.

Standard tests

Valet *et al.*, elsewhere in this issue¹, use a representation similar to Tric *et al.*², in that a single weighted mean VGP longitude is assigned to each path to avoid the bias towards transitions with large numbers of VGPs. Using standard χ -square tests they conclude that different records of the same reversal do not have a common VGP transition path, and that there is no robust evidence for a preferred longitude sector for VGP paths from different reversals.

The major problem here is a paucity of data. Using clear selection criteria, Valet and colleagues have accepted only 39 individual records, 25 of these being from five reversals younger than 2 million years and the remaining 14 from reversals younger than 12 million years. However, their message is clear: the data do not at this stage demand a preferred longitude sector, for it is not implausible that the observed distribution of paths could have been obtained from a uniform random distribution. Clear resolution can come only with a substantial increase in the database, which is probably quite achievable, but will not be immediately forthcoming.

There is obvious attraction in the idea that the Earth's mantle exerts a direct control over the behaviour of the core during a reversal, but Valet *et al.* have shown that the hypothesis is not yet proven. □

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A champion thallus

Clive Brasier

ALTHOUGH fungi have acquired notoriety as agents of disease and as producers of psychedelic toadstools, their vegetative structure, the mycelium or fungal thallus, has a somewhat lower public profile. Wider recognition of these structures and their part in nutrient cycling, not least from ecologists, is likely to come from the report by Smith and colleagues on page 428 of this issue¹. Smith *et al.* describe a thallus of the tree-root colonizer *Armillaria* which they estimate is more than 10,000 kg in weight and some 1,500 years old.

Fungal mycelia are typically made up of networks of foraging tube-like hyphae. In the higher fungi such as ascomycetes and basidiomycetes, adjacent hyphae of the same species have a strong propensity to fuse. In consequence, many mycologists once considered the thalli of these fungi as physiologically cooperative mosaics, regardless of whether the component hyphae were genetically similar or dissimilar. But support for altruism has waned, and fungal thalli have been shown to conform to the modern evolutionary dogma of the selfish gene. Genetically different mycelia are often mutually antagonistic rather than cooperative, and attempted hyphal fusions between them usually break down^{2,3}. This ability to distinguish mycelial self from nonself is regulated by multiple locus, multi-allelic genetic systems. Like the human histocompatibility system, these can generate large numbers of different recognition types, termed somatic or vegetative compatibility types, which identify different genetic individuals. Across the fungal world, these vegetative compatibility systems not only maintain a mycelium's territory, but are also involved in inter-territory invasion, in influencing sexual out-

crossing potential, and in restricting the spread of foreign DNA or RNA, such as mycoviruses, plasmids, nuclei and mitochondria, between adjacent mycelia. Through interplay with the mating systems that allow sexual exchange between individuals, they orchestrate the genetic structure of fungal populations.

Smith *et al.* used the 'random amplified polymorphic DNA' (RAPD)

Although individual thalli of spore-dispersed fungi are often small, many species produce clones, mainly by asexual sporulation, which may become geographically widespread or dominant. This restricted diversity may reflect specialized breeding behaviour or unusual evolutionary circumstances, or may simply result from chance. In some species clones are a product of an exclusively asexual mode of reproduction or reflect the predominance of inbreeding. That the wheat rust, *Puccinia striiformis*, is near clonal world-wide⁷, may reflect a loss of its sexual stage and strong host



FIG. 2 Three *Armillaria* root-disease centres in virgin coniferous forest in Montana. The lower (circular) centre covers nearly 8 hectares. (Photo by Ralph E. Williams.)

approach, together with analyses of vegetative compatibility and mating type, to assess the extent of genetic individuals in an *Armillaria* thallus (one of a growing number of applications of RAPDs in ecology, as discussed in these pages earlier this year⁴). Similar studies have shown that in those fungi that regularly outcross, the structure of local populations ranges broadly from very

large numbers of genetic individuals in a small volume of substrate, as in the case of *Ophiostoma novo-ulmi*, the Dutch elm disease pathogen, in elm bark (a in Fig. 1), to single, often extensive individuals distributed across a forest system⁵, as can be the case with *Armillaria* species (b in Fig. 1). Fungi of the first type tend to arrive at or leave a food source mainly by means of spores, whereas those of the second, once established, will also locate fresh food sources by means of exploratory vegetative growth⁶.

selection imposed by intensive wheat breeding. In *O. novo-ulmi*, abundant genetic individuals may be the norm, but current epidemic fronts are dominated by clones whose existence seems to reflect a combination of founder effects and higher fitness. Later these frontal clones are replaced by a highly heterogeneous population structure, a change that is apparently driven by the spread of debilitating virus-like diseases within the clones⁸.

Among the vegetatively exploratory fungi, potential longevity increases the chances of dominant individuals emerging, particularly in comparatively stable ecosystems. Thus in *Armillaria*, which produces networks of melanized hyphal bundles called rhizomorphs within the forest floor, infection centres of 300–500 m across comprising only one or a few genetic individuals are not uncommon, although the size of individuals may be influenced by forest conditions and species strategy as well as by age^{9,10}. The latest and perhaps greatest infection centre to be described is that of Smith *et al.*¹, who have identified a single genetic individual of *A. bulbosa*, 'clone 1', occupying some 15 hectares. Despite

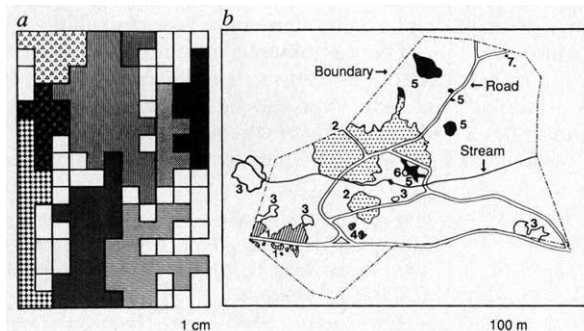


FIG. 1 a, Distribution of 39 genetic individuals of *Ophiostoma novo-ulmi*, the pathogen causing Dutch elm disease, in an 11×16 cm piece of elm bark. Each square, rectangle or shaded portion represents a unique genotype¹². b, Distribution of seven genetic individuals of *Armillaria luteobubalina* in a eucalypt forest in the 33-hectare Victoria Mills Scenic Reserve, Victoria, Australia⁹.

clone 1's estimated 1,500 years of existence, the authors' preliminary molecular analyses suggest that it has remained genetically highly conserved across its enormous biomass. Given the likelihood of mutation, sexual and somatic nuclear recombination, and more novel if rare genetic events described for similar fungal systems, the genetic stability of the clone is indeed remarkable.

Other matters concerning the stability of the thallus remain to be settled. Is the establishment of such an infection centre a rare event, and has the persistence of clone 1 in a fluctuating environment been a matter of chance, or does it possess a better than average set of fitness attributes? And has clone 1 supplanted adjacent, lesser individuals during its 1,500-year lifetime? At least it should be around long enough for some of these questions to be answered.

The suggestion of Smith *et al.* that clone 1 deserves recognition as one of the largest of living organisms, rivalling the blue whale or the giant redwood, invites closer scrutiny. The blue whale and redwood exhibit relatively determinate growth within a defined boundary, whereas fungal mycelia do not. Substantial rhizomorphic systems such as those of clone 1 may also expand and regress considerably in response to the local availability of food sources, and to events such as fire, climatic fluctuation and forest succession. Furthermore, after 1,500 years, clone 1 is likely to have become fragmented into a number of independently functioning components, as demonstrated by another study (*b* in Fig. 1) in which large *Armillaria* clones were shown to have become physically disjunct. The clone's structure may be more akin to that of some vegetatively reproducing plants such as grasses¹¹ and bracken, and some ancient elms. So although clone 1's reputation as a champion genotype may yet be secure, its status as a champion organism depends upon one's interpretation of the rules. □

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Back to the drawing board

Peter W. Stephens

In the short time since the discovery of superconductivity in doped C_{60} fullerenes, a simple relationship has emerged between the size of the crystalline unit cell and the superconducting transition temperature. This important result has guided the search for variants with higher transition temperatures and has acted as an important ingredient in models of the superconductivity. Now, according to two papers in this issue^{1,2}, new doped fullerenes have been synthesized which violate the simple rule. Inasmuch as one learns more from the violation of a rule than from its establishment, this result will surely stimulate new insights into these intriguing superconductors.

Data on superconducting fullerides, with the chemical formula A_3C_{60} (where A is an alkali atom), have been accumulating for a year and point the way towards a simple correlation between structure and superconductivity: the larger the lattice spacing (a_0), the higher the superconducting transition temperature (T_c). It was first discovered³ that T_c in K_3C_{60} and Rb_3C_{60} plummeted as pressure is applied to the samples. A similar observation was made⁴ by comparing fullerenes doped with mixtures of K, Rb and Cs. All have the same structure, but the larger alkali ions push a_0 to larger values. A plot of T_c against a_0 shows a steady increase of about 55 K per Å. (Nature always reserves the right to terminate such an encouraging empirical relationship, and indeed it does not

appear to be possible even to prepare the end point of that series, Cs_3C_{60} .)

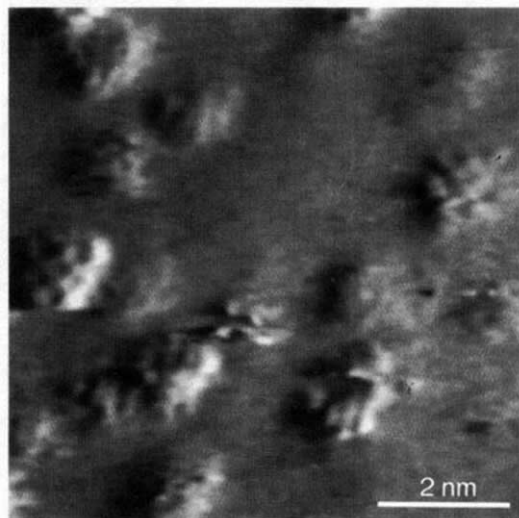
The two sets of observations were brought together only recently by measurements of the compressibility of fullerides⁵. A compilation of the pressure and cation alloy data shows that for K, Rb and their mixtures (including those with Cs), the lattice constant governs the superconducting transition temperature, with the specific cations having only a minimal effect.

In general, the relationship between the atomic structure of a solid and its superconductive properties is not a simple issue; even within a particular family of superconductors, there are enough uncontrolled factors in forming new varieties that it is unusual to find a trend that can be exploited with confidence. For example, although it is generally the case that the transition temperature of high- T_c cuprate superconductors rises as more intervening layers of atoms are inserted between the all-important CuO planes, numerous exceptions have frustrated the design of materials with a higher T_c by this rule.

Another example is afforded by Nb_3Sn , which is used commercially in very-high-field superconducting magnets. Although dozens of members of this family are known, it is a difficult job to correlate their superconductive properties with structural information. Indeed, of all the known superconducting materials, the alkali-doped fullerenes provided the best opportunity of corre-

C_{60} structure brought into view

THE soccerball shape of C_{60} is possibly more familiar now than any other molecular structure, apart perhaps from the hexagon of benzene or the double helix of DNA. Yet only now is it being seen directly. This image, taken with a scanning-tunnelling microscope, just reveals the internal arrangement of individual C_{60} molecules attached to the surface of a gold crystal (Y. Zhang, X. Gao & M. J. Weaver *J. Am. chem. Soc.* **96**, 510–513; 1992). The interconnected rings of each molecule are just apparent; in some cases the central (uppermost) ring is surrounded by five other rings, in others six — reflecting the mixture of five- and six-membered rings of the fullerene. The authors suggest that they can see the internal structure because the molecules become wedged into deep ruts on the surface of the gold. On other surfaces, the molecules are free to rotate on timescales far short of the few seconds it takes a scanning microscope to complete an image, erasing any detail.



M. J. Weaver

R.P.

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In the dock

EVERY desert scene comes with scorpions, but these rugged animals are found in various habitats. T. G. Benton has been studying the thriving population of *Euscorpis flavicaudis* which has been resident in the dockyards of Sheerness in southern England, possibly since Victorian times (*J. Zool., Lond.* **226**, 351–368; 1992). Benton made 10,000 sightings of the creatures, and estimates that the population is close to 700. But the animals are highly reclusive, spending almost all their time hidden in cracks in the dock walls (females, for instance, leave their holes on average fewer than ten times a year). Otherwise they behave very much as if they lived in the desert. Scorpion lifestyle is hardly influenced by abiotic factors such as temperature: this, suggests Benton, is the key to their adaptability.

Where the ions are

PROTEINS are polyions, bristling with charged groups at their surfaces, the electroneutrality of which is maintained by counterions. To what extent these occupy unique structural sites, rather than being merely more concentrated within the protein domain, is a question that has agitated chemists since Donnan. Crystallography gives the means of resolving the riddle and O. Gursky *et al.* (*Biophys. J.* **64**, 604–611; 1992) have tackled it by reference to one of the smallest of globular proteins, insulin. Sodium ions cannot be distinguished by scattering from water and Gursky *et al.* replaced them by other univalent cations, in particular thallium. Two site-bound cations were found, one in an enclosed cavity, the other trapped between two insulin molecules, and each interacting apparently with a pair of carbonyl dipoles. The greater part of the neutralization comes from counterions at large in the solution.

Light spin

Physicists are becoming adept at manipulating atoms and molecules in beams of laser radiation. Optical molasses cool atoms through viscous friction; and optical tweezers trap atoms with light pressure. Another concept of fluid mechanics creeps into these studies with the report from A. Hemmerich and T. W. Hänsch of optical vortices (*Phys. Rev. Lett.* **68**, 1492–1495; 1992). The authors fired a beam of rubidium atoms through the intersection of a pair of crossed laser beams. With the beams in phase, the atoms emerged unscathed, the radiation pressure being isotropic. With the lasers out of phase, off-axis pressure vortices pulled the atom beam's edges visibly out of shape. The authors expect that optical molasses, with three lasers and special polarization conditions, could give highly unusual effects.

lating structure with properties.

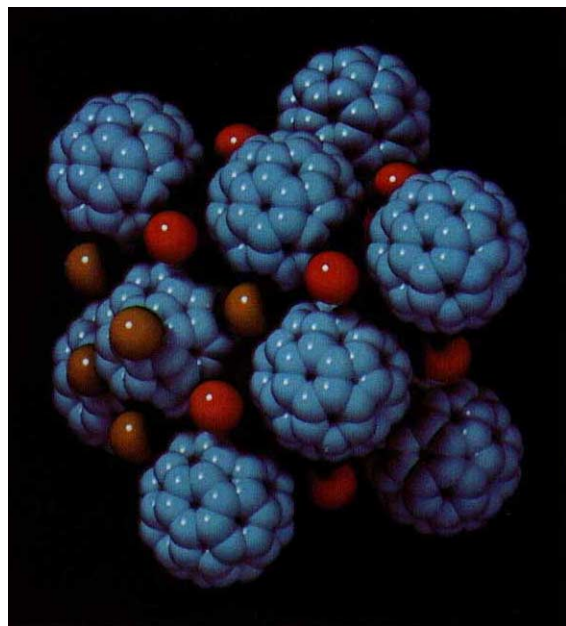
The fact that a_0 rigorously controls T_c was easily explained (once observed) by nearly every model of superconductivity. Any theory of superconductivity starts with some mechanism, exotic or prosaic, to create a condensate of paired electrons, which can carry a supercurrent through the lattice. Independent of the precise specification of that mechanism, the rules of weak-coupling 'BCS' theory of superconductivity hold that the transition temperature rises sharply as the number of electrons able to carry the supercurrent (those at the 'Fermi' energy) increases. As the lattice expands, there is less quantum-mechanical overlap and less interaction between electrons on adjacent fullerenes and the energy spanned by the electronic band becomes narrower; consequently more electrons find themselves near the Fermi energy. (Unfortunately, it is not obvious that T_c could continue to rise for bandwidths less than the electron pairing energy.) So the fact that T_c is inversely related to a_0 does not reveal anything directly about the particular mechanism of superconductivity. Other experiments, such as the variation of T_c with isotopic substitution, may do so, but the dust has not yet entirely settled.

This simple state of affairs has been subverted by the discoveries reported in this issue. Workers at AT&T Bell Laboratories, who report on page 416¹, have doped fullerene with the smaller alkali, Na, and found no sign of superconductivity. This material has a value of a_0 which would have led one to expect superconductivity at T_c around 16 K; we have clearly fallen off the established curve of T_c against a_0 . The authors find a reason for this disruption, in that the Na_3C_{60} apparently disproportionates into Na_2C_{60} and Na_6C_{60} as it is cooled. Indeed, recent photoelectron-spectroscopic measurements partially anticipated this result by showing that both Na- and Li-doped fullerenes are insulators across the entire range of compositions⁶.

In a related article, on page 419², a group from NEC Fundamental Research Laboratories, Japan, explores material doped with mixtures of Na, Li and heavier alkalis. The researchers find that these materials do superconduct, but with T_c values far below what is expected from their lattice constants. For example, $\text{Na}_2\text{RbC}_{60}$ has a T_c of 2.5 K, whereas K_3C_{60} compressed to the same

a_0 of 14.028 Å becomes superconducting at 10.5 K. Both papers show that the inclusion of smaller alkali ions actually compresses a_0 to below that of the undoped C_{60} . It appears that such an internal compression is strongly inimical to superconductivity.

The time also seems right for stuffing an unexpected excess of cations into fullerene crystal lattices. Previously known cubic-close-packed fullerenes have the stoichiometry A_3C_{60} , with one alkali ion in each interstitial space of the



Space-filling model of the doped fullerene superconductor K_3C_{60} , showing the extra space available to cations in octahedral vacancies (red).

array of fullerenes (see figure). A_4C_{60} and A_6C_{60} , for $\text{A} = \{\text{K}, \text{Rb}, \text{Cs}\}$ have different lattice structures. But sodium gives the same cubic-close-packed structure for stoichiometries from Na_2C_{60} all the way up to Na_6C_{60} (ref. 1). A second group at AT&T Bell⁷ has found that the alkaline earth Ca can produce superconductivity in a cubic-close-packed phase with a very high concentration of Ca, around Ca_5C_{60} . The octahedral vacancy site in the lattice of fullerenes is somewhat larger than a single alkali ion. But with Na_6C_{60} and Ca_5C_{60} , one would have to cram several cations into that vacancy. It is a challenge to imagine how Na–Na distances as small as those described in this issue by the first AT&T Bell group could arise.

So we find the empirical relationship between a_0 and T_c becoming shaky, along with its simple implications. Once researchers have had a chance to sort

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2. Tanigaki, K. *et al.* *Nature* **356**, 419–421 (1992).
3. Sparr, G. *et al.* *Science* **252**, 1829–1831 (1991).
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7. Kortan, A. R. *et al.* *Nature* **355**, 529–532 (1992).

J. Lauer & P.W.S.

through this expanded bestiary of fullerene superconductors and also-rans, some stronger understanding of the relationship between structure and superconductivity may emerge. At the same time, the discovery of variations to the theme keeps open an optimism that CLIMATOLOGY

there may be useful, instead of merely interesting, fullerene superconductors waiting to be invented. □

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Molecules record sea change

Mitchell Lyle

LONG-CHAIN (C_{37} – C_{39}) alkenones are organic molecular fossils from a class of phytoplankton which can be used to create a record of sea surface temperature from marine sediment deposits. Eglinton *et al.* on page 423 of this issue¹ have used this technique to produce a 650,000-year record of sea surface temperature from an upwelling area off northwest Africa. They examine in detail three climatic transitions from glacial to interglacial conditions and find not only strong effects related to the Earth's orbitally-caused changes in solar heating (Milankovitch orbital forcing) but also intriguing higher-frequency cyclicity which, if it exists, may reflect harmonic responses internal to Earth's climate system.

How the long-chain alkenones record environmental temperature is not completely known, although the correlation between sea surface temperature and the proportion of unsaturated forms of these compounds is undeniable^{2–4}. An index ratio (U_{37}^k) of di-unsaturated to di- and tri-unsaturated forms of the C_{37} alkenone has been calibrated to growth temperatures in culture of the coccolithophorid algae *Emiliana huxleyi* and to sea surface temperature for environmental samples⁴. There has been speculation — because of the alkenones' temperature response, their chain length and their overall abundance in living cultures (about 8 per cent of the total carbon in the phytoplankton⁵) — that they are cell-membrane lipids. If this speculation is true, the degree of unsaturation of these compounds could have a direct physiological link to environmental temperature — the cell changes the degree of unsaturation to maintain a constant viscosity of the membrane. Indeed, Prahl *et al.*⁵ note that, in culture, living algae respond in a matter of days to changing temperature conditions.

Because the cells adjust rapidly, however, it becomes important to establish what environmental temperature is being recorded by the phytoplankton. Many of the algae concerned live not right at the surface, but down to a depth of 100 metres, in the euphotic zone⁶. As long as the euphotic zone approximately

matches the thickness of the ocean's surface mixed layer, the temperature recorded by the alkenones can be reasonably interpreted as sea surface temperature. Care must be taken when interpreting the U_{37}^k data, however, where strong temperature transitions occur in the upper 100 metres of the ocean. In addition, most phytoplankton also have seasons of maximum growth, and the temperature recorded by the alkenones in sediments should represent primarily the sea surface temperature during blooms, when most of the carbon is incorporated into the biomass.

Eglinton *et al.*¹ study a core collected by the Ocean Drilling Project at the southern edge of one of the main upwelling cells off subtropical northwest Africa, at Cape Blanc. The region is ideal for an investigation of this sort — the modern sea surface temperature over the site is relatively stable all year round (18–22 °C) and is controlled by tradewind-induced coastal upwelling⁷. Typical subtropical waters surrounding the upwelling cell are several degrees warmer than this, so that the sea surface temperature here is directly linked to northerly wind strength.

Eglinton *et al.* have several important features in their record. The modern U_{37}^k temperature estimate falls midway in the annual range of sea surface temperatures, and seems to be near the annual mean for the location. The U_{37}^k temperature record may thus reflect average annual upwelling at Cape Blanc. Over the 650,000 years of their record the authors observe a maximum temperature cycle of 9 °C and typically observe 5–6 °C warmings tied to deglaciations of the Northern Hemisphere ice sheets.

Because the Canary islands, which block the southward flow of water along the coast today⁷, probably also blocked flow during glacial climate periods, there is little likelihood that these temperature changes are due to advection of cold northern waters. Instead, they must reflect higher upwelling of subsurface waters during glacial climate states and imply that tradewind speeds at Cape Blanc must have been higher on average in glacials than interglacials. Although Eglinton *et al.* attempt to link the

temperature changes they observe to changes in North Atlantic deepwater formation, the connection between the two processes is probably indirect and involves atmospheric rather than oceanic processes.

Eglinton *et al.* put most of their effort into three separate intervals of deglaciation, about 339,000, 127,000 and 12,000 years ago. They find that each is characterized by short-period events (less than a few thousand years in length) in which sea surface temperature oscillated by as much as 3 °C, or about half the total amplitude of the full change from glacial to interglacial conditions. Such large oscillations imply significant changes in global climate independent of Milankovitch-type insolation cycles.

There also seems to be periodicity in these temperatures changes — Eglinton *et al.* claim to observe significant cycles about 600 years in length. If further examination confirms this claim, there are important implications for our understanding of global climate and climate change.

Climatic periodicity on timescales shorter than the orbitally driven Milankovitch cycles (main periods of 19,000, 23,000, 41,000 and 100,000 years) have been observed previously, but data for such cyclicity are sparse. Previous glacial studies^{8,9} note significant cyclicity with a period near 2,600 years and possible indications of shorter-period variations. The 2,600-year cycles seem to be correlated with cycles in radiocarbon activity, which in turn may reflect changes in radiocarbon production due to short-term changes in sunspot activity and insolation or changes in terrestrial reservoirs or radiocarbon due to ocean mixing. Alternatively, the radiocarbon and climate cycles may be independent and the short-period climate variations could be due to feedbacks (resonances) internal to Earth's climate system⁹.

While Eglinton *et al.* observe periodicity in their record similar to that seen previously, their 600-year-old periodicity should not be due to insolation. The initial observations they report deserve to be confirmed and followed up with care, because the most likely cause for such a periodicity is an internal climatic feedback. If the timescale is correct, the length of the cycle implies that the feedback involves a coupling between

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atmosphere and the oceans most probably involving oceanic thermohaline circulation patterns (density-driven circulation, rather than wind-driven).

At present, we know very little about the scale of natural variations in thermohaline circulation. Nor do we know how those variations may affect global heat transport. We do know that large changes in thermohaline flow were associated with the last deglaciation. Study of shorter-term variations is particularly difficult, however, because the timescales involved are typically longer

than anything modern historical records can delineate, but are short enough for it to be hard to find environmental records with sufficient time resolution. More detailed study at the site investigated by Eglinton *et al.* may provide new ways to investigate global climate change, and to alleviate our present ignorance about the long-term interactions between atmosphere and oceans. □

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ANTIGEN PROCESSING

A new presentation pathway?

Alain Townsend

TWO reports — one by Henderson *et al.* in *Science*¹ last month, the other by Wei and Cresswell on page 443 of this issue² — document the binding of peptides derived from defined signal sequences to the HLA-A2 class I molecule; signal sequences are hydrophobic stretches of amino acids, often found at the amino termini of glycoproteins, that are required for protein translocation into the lumen of the endoplasmic reticulum (reviewed in ref. 3). Together with a demonstration that addition of a signal sequence to a short peptide can bypass the defect in antigen presentation in the mutant cell T2 (ref. 4), these results show that peptides capable of binding to class I molecules can enter the endoplasmic reticulum by the classical signal-dependent mechanism, and that they may be derived from the signal peptide itself. The binding of signal sequences to HLA-A2 has therefore been heralded as “A second pathway of antigen presentation” (ref. 1). However, most signal-derived peptides bound to HLA-A2 are longer than the nine residues found in HLA-A2 isolated from non-mutant cells⁵, and given the additional evidence provided by Wei and Cresswell it seems that HLA-A2 may be unusual in its capacity to bind such hydrophobic sequences.

Antigen presentation is the mechanism by which cells expose fragments of their proteins, bound to class I or class II molecules of the major histocompatibility complex (MHC), for inspection by circulating T lymphocytes. The class I molecules are known to bind peptides derived from the degradation of cytosolic proteins, and may have evolved to alert the immune system to the earliest phase of viral infection. Neither the peptides themselves nor the cytosolic proteins from which they are derived, require signal sequences for translocation into the compartment (almost certainly the endoplasmic reticulum) where

they bind to class I molecules. These observations suggested that a specialized, signal-independent transporter for peptides should exist.

A variety of mutant cells⁶⁻⁹ — including the T2 cells, a derivative of .174 (refs 6,7), that are the focus of the papers under discussion — have been identified that are unable to assemble stable complexes of class I heavy chain with β_2 -microglobulin (β_2m) and also have a defect in the presentation of cytosolic proteins to T cells^{10,11}. Both defects could be explained by mutations in the postulated peptide transporter if bound peptide were required to maintain class I molecules in the assembled state¹⁰. The effects of synthetic peptides on class I folding and assembly (reviewed in ref. 12), and the reversal of the complete mutant phenotype by transfection with the MHC-encoded transporters¹³⁻¹⁶, is consistent with this view. (Note that T2/.174 has deleted both transporters and as yet has not been complemented by transfection.) The virtual absence of peptides detected on HLA-B5, HLA-A3, H-2K^b and H-2D^p in T2 cells reported by Wei and Cresswell², and the restoration of antigen presentation in T2 by the agency of a signal sequence⁴, also strongly point to a defect in signal-independent transport.

But some data appear to require extension of this simple view of the mutant phenotype (not all of which can be reviewed here¹⁷). Cresswell and colleagues have been interested for some time in the factors that control expression of class I molecules at the surface of the mutant cell .174/T2. He and Wei now show that although they contain minimal quantities of peptides, the murine class I heavy chains D^p and K^b are expressed at normal levels on T2 cells. In contrast HLA-B5, which also contains no detectable peptide, is retained in the endoplasmic reticulum.

The results establish first that murine

heavy chains associated with human β_2m can form stable heterodimers without peptide, probably because the dissociation rate of human β_2m is slow enough to allow accumulation of the complex at the cell surface. These heterodimers should make admirable material for crystallization. Second, they show that there is a mechanism for retaining certain peptide-free class I molecules in the endoplasmic reticulum. The most reasonable explanation is that the retention mechanism distinguishes between human and mouse class I heavy chains that lack peptides, but is not sensitive to the stability of association of the heavy chains with β_2m .

Thoughts along these lines led both Henderson *et al.*¹ and Wei and Cresswell² to investigate the unexpected behaviour of HLA-A2, which is the only human heavy chain known to pass out of the endoplasmic reticulum of T2 cells and accumulate (at variable, but low levels) at the cell surface^{6,7}. The finding that HLA-A2 can bind signal sequences clearly explains why it may leave the endoplasmic reticulum. The sequences of the bound peptides revealed the expected binding motif for HLA-A2 described recently⁵, and confirmed in the two new papers. This suggests that the binding is specific and related to the hydrophobic nature of the specificity pockets in the HLA-A2 binding site. The almost complete lack of peptides bound by HLA-B5 and -A3 implies that the relatively limited diversity available in signal sequences is unlikely to give rise to peptides that can be bound by most human class I molecules. However, the HLA-A2 is the most common class I allele in the human population, and therefore the potential importance of this mechanism should not be underestimated.

A point not stressed in these reports is that, despite the bound peptides, the majority of HLA-A2 molecules isolated from .174 or T2 cells are unstable (dissociate readily *in vitro*) but can be stabilized by excess peptide^{11,18}. An explanation for this instability may lie in the length of the peptides bound in the endoplasmic reticulum of T2 cells. Most of them are longer than the nine residues found in HLA-A2 from non-mutant cells⁵. Measurements of the effect of length on the dissociation rates of peptides from the H-2D^b molecule expressed in T2 showed that any addition beyond the carboxy-terminal ‘anchor’ residue increased the rate of dissociation of the peptide some 100 times¹⁹. When aligned with the known anchor residues, most of the peptides bound in T2 are extended at both ends, and would therefore be expected to dissociate rapidly at the cell surface in conditions of infinite dilution of the peptide.

EARTHQUAKES

Seismic gaps and grizzly bears

Seth Stein

Whether this will prevent presentation to T cells remains to be tested.

The length of the HLA-bound peptides in T2 puts a strain on the hypothesis of Falk *et al.*²⁰ that peptides of more than nine residues in length may enter the endoplasmic reticulum, bind class I, and then be trimmed to the stable binding length by specialized enzymes resident in the endoplasmic reticulum. Why didn't this happen to all the signal peptides in T2? One possibility is that these hypothetical trimming enzymes are encoded within the segment of the MHC deleted in T2 cells. This, however, is ruled out by the application of the extraordinarily sensitive technique developed by Hunt and colleagues²¹ of combined microcapillary high-performance liquid chromatography and mass spectrometry to analyse the peptides bound to HLA-A2 in normal T1 cells. All of the long signal-derived peptides found in T2 were also found in T1.

The implication here is that trimming of peptides in the endoplasmic reticulum is not efficient if the peptides have entered by the signal-dependent route. Three speculative alternatives to the original trimming hypothesis can be proposed (see also refs 1 and 2). First, the trimming mechanism may occur as originally envisaged in the endoplasmic reticulum, but require the MHC-encoded transporters as subunits. Second, the template for the trimming mechanism may be the binding site or internal lumen of the peptide transporter rather than class I. Finally, the proteolytic machinery in the cytosol may be specialized at cleaving proteins into defined lengths before their transport into the endoplasmic reticulum. □

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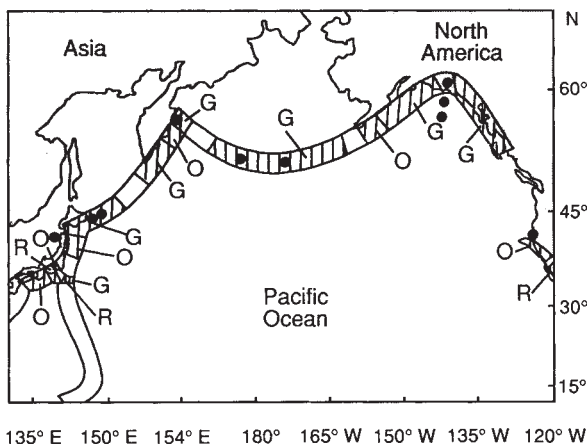
EARTH scientists joke that data mess up perfectly good theories. The widely accepted seismic-gap hypothesis, which underlies most attempts to predict earthquakes, has just been tested by Kagan and Jackson¹, and falls, they say, to this regrettable fate.

Most large and great earthquakes (with magnitudes greater than 7 and 8, respectively) occur along the boundaries between plates. Although motion between plates occurs steadily, as shown by the agreement between plate motions

been assumed to apply to other segments of plate boundaries, even where the historical record is too short or too incomplete to indicate a quasiperiodic cycle. The hypothesis is crucial in studies working towards earthquake prediction. Such studies originally focused on short-term predictions (for events to occur in less than a few years) and were based on properties such as seismic velocity, which had been reported to change observably before earthquakes. But such changes were not observed generally, even before large earthquakes in well-monitored areas, and attention turned to long-term forecasting of probable earthquake locations. The gap hypothesis permits probabilistic estimates with the assumption that the longer the time since a major earthquake at a given location, the higher the probability that one will soon occur^{4,5}.

The most widely used of such estimates are from a map developed in 1979 by McCann, Nishenko, Sykes and Krause, who divided plate boundaries into segments⁶. They defined segments that had not experienced large earthquakes in the past 30 years as seismic gaps and characterized certain gaps as having a high seismic potential for a large earthquake in the next few decades. Some large earthquakes have occurred in the high-potential gaps, and have been interpreted as evidence for the validity of the approach⁷.

Kagan and Jackson have now conducted the first rigorous test¹ of the hypothesis, noting that it “cannot be fairly evaluated by considering only successes; even if a few randomly selected seismic zones were labelled as gaps, some earthquakes would occur in them purely by chance. If the seismic gap model is valid, it should be able to predict future earthquakes better than a purely random scheme”. They consider the 37 earthquakes with magnitude 7 or greater that occurred in the ten years after McCann *et al.* published their map in zones to which McCann *et al.* assigned a seismic potential. Of these, 4 (11%) occurred in the postulated high-potential seismic gaps, 16 (43%) in zones of predicted intermediate potential, and 17 (46%) in zones assumed to have low potential (for example, see figure). The



Seismic risk on the North Pacific rim as postulated by McCann *et al.*⁶ in 1979. Shaded segments of the rim were assigned seismic potential (R, red = high; O, orange = intermediate; G, green = low); unshaded segments, potential uncertain. During the ten years that followed the map's publication, ten large earthquakes (black circles) occurred. None occurred in the high- or intermediate-risk areas, and five in the low-risk segments. (From ref. 1.)

averaged over millions of years and those observed over a few years by space-based geodetic measurements, motion along the boundaries generally occurs episodically in large earthquakes. It is thus natural to think of a seismic cycle over which the strain due to the plate motion accumulates and is released by earthquakes. One can then define seismic gaps, areas along the plate boundary which have not recently ruptured in a major earthquake, and hypothesize that these are likely sites for future earthquakes.

The gap hypothesis has been generally accepted for several reasons. It extends a classic concept, first proposed following the 1906 San Francisco earthquake. Earthquakes would be expected to be quasiperiodic if they occur when the stress on a fault reaches a characteristic value². Most importantly, historical accounts indicate a pattern of recurrent earthquakes along some segments of plate boundaries³.

As a result, the gap hypothesis has

postulated high-potential gaps thus have been less seismically active than those with lower potential. This is not an artefact of region size, because the net areas of high-, intermediate- and low-potential regions differ by only 10%.

Kagan and Jackson apply several statistical tests to the gap hypothesis. They try a model in which earthquakes were twice as likely to occur in high-potential as in low-potential regions, and find that the data permit the hypothesis to be rejected at a confidence level above 95%. They similarly conclude that more than 90% of maps generated by assigning seismic potential to each zone randomly would predict the earthquake locations better than that of McCann and colleagues.

The apparent failure of the gap model is surprising, given its intuitive good sense. A natural question is whether the model is valid, but applicable only to larger earthquakes. Such tests are difficult, because the larger the magnitude, the rarer the earthquake, so earthquakes of magnitude 7 or greater are roughly ten times more common than those of magnitude 8 or more. Kagan and Jackson examine earthquakes over their ten-year period with magnitudes in excess of 7.5, and find one in the high-potential zones, three in the medium-potential zones, and five in the low-potential zones. So although the sample size is smaller, the gap model still seems unsuccessful.

Alternative

The alternative to the gap hypothesis is that earthquakes, rather than being quasiperiodic, occur in clusters. Kagan and Jackson consider the ratio of the standard deviation of the time between earthquakes to the mean time between them. If this ratio is less than 1, the process is quasiperiodic, whereas clustering would give a ratio greater than 1. Clustering has been suggested for the southern San Andreas fault, where over the past 1,500 years the mean period between events is 132 years with a nominal standard deviation of 105 years⁸. If earthquakes do cluster, the gap assumption of reduced seismicity after a large earthquake would not apply, presumably because not enough accumulated strain is released to preclude subsequent earthquakes.

Kagan and Jackson's results suggest that earthquake recurrence should be re-examined critically. Even if the gap concept proves to apply for great earthquakes, its utility for hazard assessment will be limited if it is inapplicable to smaller earthquakes which are still destructive. It may be that seismicity in some regions is quasiperiodic, whereas in others it clusters. The apparent failure of the gap model should thus spur fur-

ther work in studies of fault processes. One key area is the interaction in space and time between slip on adjacent portions of faults^{4,9}. There is also much to be learned about the fraction of the plate motion that does not occur as seismic slip: this fraction varies dramatically between plate boundaries¹⁰ and can pose difficulties for identification of seismic gaps and estimates of recurrence times¹¹.

Assumptions

Kagan and Jackson's new study will do much to shape thinking on earthquake prediction. It demonstrates the need to pose forecasts in a form suitable for statistical testing, and to test them against subsequent data. For example, it would be meaningless to state that the gap hypothesis predicts only gap-filling earthquakes. Similarly, the authors note the need to test whether the observed quasiperiodicities at some plate boundaries exceed those expected purely by chance. Such testing, together with analysis of key assumptions used in forecasts¹², should help establish how useful probabilistic earthquake forecasts are. Because earthquakes on individual plate-boundary segments are infrequent by human timescales, centuries may be needed to provide a consensus.

Until then, inferences will still have to be drawn largely from the non-occurrence of earthquakes, a challenging prospect because *a priori* assumptions must be made. By way of analogy, fatal attacks by grizzly bears are more common in Montana than in New York. Does this indicate a 'bear gap' in New York so that an attack is now more likely there? Or does it represent a difference in intrinsic hazard? Depending on the assumptions made, either interpretation is tenable. □

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Icy blasts

A SPACE rocket needs to pack as much energy as possible into a given mass of fuel. Hydrogen and oxygen are about the best of stable fuels. Propellant chemists look longingly at such unstable substances as monatomic hydrogen, and the hydride and hydrogen-molecule ions, which would give maybe thirty times as much energy if only they could be captured. Daedalus now plans to make them by irradiating solid hydrogen.

He has been inspired by the 'matrix isolation' of unstable molecules. This technique incorporates suitable precursor molecules in a matrix of solidified gas near absolute zero, and then irradiates it with ultraviolet light. The molecules are created and trapped *in situ*. Daedalus calculates that ultraviolet light of wavelength 270 nm should split hydrogen molecules into single atoms. A wavelength of 70 nm could divide them into hydride and hydrogen ions. High-energy radiation could induce more drastic reactions still.

Solid hydrogen, of course, is merely the minimum-energy arrangement of equal numbers of protons and electrons. All possible reactions within it simply shift these charged entities into more energetic positions. Daedalus will encourage these shifts by maintaining a strong electric field across his frozen hydrogen. As the incoming radiation springs the particles out of their original sites, the field will prevent them just falling back in a recombination reaction. It will flick them away to lodge elsewhere. The final nightmarish product should be the most energetic distribution of protons and electrons that can be just stabilized by a temperature near absolute zero. It may still contain eighty-five per cent of normal hydrogen, separating and stabilizing its contents of high-energy atoms, ions and sites. This could still give it six times the energy of hydrogen-oxygen fuel (and twenty times that of TNT).

The result will be a bomb of appalling violence, ready to explode the moment its temperature rises the few degrees to its melting point. Even Daedalus does not recommend it for primary lift-off. But as a sustainer for long space journeys it is ideal. A spacecraft can call on sunlight or nuclear power for its energy; but its propellant mass is limited by what it can carry itself. A probe carrying pure hydrogen could use its power supply to freeze the gas and matrix-irradiate it into little pellets of hyper-energetic fuel. By exploding these at intervals in an expansion nozzle, it could generate enough thrust to drive around the Solar System as never before. Undignified expedients like swinging round Jupiter to gain speed might no longer be needed. David Jones

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Mercury pollution in Brazil

SIR — Gold mining in the Brazilian Amazon involves more than 650,000 people and results in the annual discharge of 90–120 tonnes of mercury into local ecosystems^{1,2}. Miners extend the gold rush each year to other rivers and the release of mercury into the environment of the region continues to rise. For more than ten years, the Madeira River basin has been the centre of the gold rush in Brazil and has yielded more than 100 tonnes of gold so far^{1,2}. We present probably the first accurate concentrations of mercury in the Madeira River (see table), and demonstrate that the bioaccumulation of pollutant mercury in many fish species has resulted in levels considered unsafe for human consumption. Many miners and some local residents may already be poisoned by mercury³, but the effects of the ongoing buildup of mercury in local food chains may become more severe on wildlife, especially piscivorous mammals.

Our study was conducted in the State of Rondonia, between the towns of Porto Velho and Guajara Mirim near the Bolivian border. Surface-water samples were collected in properly decontaminated polyethylene bottles using scrupulous methods in the field to exclude sample contamination. Determination of Hg in the samples was made by cold vapour atomic absorption spectrometry (CVAAS) in an ultra-clean laboratory equipped with gold-coated air filters⁴. Edible portions of many fish species from the local waters were also analysed by CVAAS. The dissolved and particulate Hg concentrations in the Madeira River and its tributary average 13.8 ± 2.5 and 10.8 ± 4.4 ng l⁻¹, respectively (see table). The particulate form of Hg thus constitutes about 44% of the mean total Hg concentration of 24.6 ± 4.5 ng l⁻¹. These results are many times lower than the previously reported Hg concentrations in the Madeira River which range

from <40 to >9,000 ng l⁻¹ (see ref. 3). By comparison, the dissolved Hg concentrations in unpolluted lakes and rivers are generally <3 ng l⁻¹ (ref. 5). The average value for Madeira River is about 17-fold higher than the 0.82 ng l⁻¹ estimated to be the mean dissolved Hg concentration in the rivers of the world⁶, and the total Hg concentrations are higher than the 4-day ambient water quality standard of 12 ng l⁻¹ that has been established by the US Environmental Protection Agency⁷.

The estimated total release of Hg in the Madeira River basin is about 32 t yr⁻¹ (refs 1, 2). From the average flow rate (920×10^{12} l yr⁻¹) and total Hg concentration, the transport of Hg by the Madeira River is estimated to be 23 t yr⁻¹, implying that most of the pollutant Hg is exported to the Amazon River, thereby extending the region of influence of the mining operation. About 28% of the Hg pollution is stored locally in the river basin, mostly in the sediments, aquatic biota, soils and vegetation. Indeed, the Hg content of some stream sediments exceeds 150 µg g⁻¹ (ref. 3), and puddles of Hg are common on soils and rock surfaces at the shores of the mining areas.

The severity of Hg contamination is well documented by the following elevated Hg levels in fish from Madeira River and its tributaries: 1.01 ± 0.64 , 0.13 ± 0.08 and 0.12 ± 0.06 µg g⁻¹ for carnivorous, omnivorous and detritus-feeding species, respectively³. The Hg contents of most of the carnivorous and some of the omnivorous species exceed the limits (<0.5 µg g⁻¹) for Hg in edible fish tissues that have been established in many countries⁸. The Madeira is a productive river and a major source of fish for local populations in its drainage basin^{1,2}. The consumption of 10–20 g of the carnivorous fish on a regular basis by a 20-kg child can easily result in mercury

poisoning considering that the World Health Organization recommended tolerable daily intake limit is only 0.43 µg per kg body weight⁸. If the child also lives near where the amalgam is burned, there is added intake of Hg from the atmosphere and contaminated dusts.

Although the popular news media have given extensive coverage to Hg pollution associated with the gold mining, scientific investigation of most aspects of the problem has been very limited. The environmental costs of the contamination of the Amazon with mercury could be enormous.

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Human origins

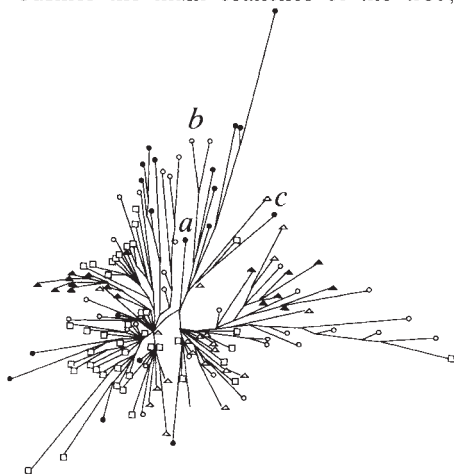
SIR — The superb sets of human mitochondrial tree data collected by Cann *et al.*¹ and Vigilant *et al.*² have turned out to be remarkably difficult to analyse in a way that can yield a consistent story about human origins. The possibility that the data show an African mitochondrial 'Eve' has recently been questioned in the light of the discovery that the apparent exclusively African branch is not as clearly separated from the rest of the tree as had originally been suggested^{3–6}.

The ways in which these and many other phylogenetic trees are usually presented pictorially may encourage inadvertent overinterpretation of the data⁷. The reasons are twofold. First, trees are usually drawn with time on the x-axis, so that the branches of the trees are arranged horizontally. Sometimes a procrustean approach is used to lengthen or shorten the branches arbitrarily and

MERCURY CONCENTRATIONS (NG L⁻¹) IN MADEIRA RIVER WATER AND ITS TRIBUTARIES

Sample location	Dissolved	Particulate	Total
Mutum Parana (junction with Madeira River)	11.3	14.5	25.8
Mutum Parana (30 m from highway)	9.8	17.5	27.3
Prainha	15.0	18.2	33.2
Humaita, mid-river	15.8	6.4	22.2
Humaita, nearshore	12.5	8.5	21.0
Porto Velho	13.7	10.4	24.1
Cachoeira Teotonio (a fishing village)	13.3	6.6	19.8
2 km above Teotonio Falls	10.7	9.1	19.9
4 km above Teotonio Falls	16.3	6.2	22.5
8 km above Teotonio Falls	17.2	8.5	25.6
10 km above Teotonio Falls	12.7	9.1	21.9
12 km above Teotonio Falls	17.1	15.3	32.4
Average	13.8 ± 2.5	10.8 ± 4.4	24.6 ± 4.5

to make them all the same length. This tends to mask inequalities in rates of substitution along the different branches. More subtly, to keep all the horizontal branches parallel, it is necessary to introduce vertical lines of various lengths. These lines, which correspond to no feature of the data, impart an artificial air of organization to the tree — the bushier the main branches of the tree,



Starburst plot of the data of Cann *et al.*¹. *a*, Position of the lone African bushman in the sample; *b*, the anomalous Asian members of the 'African' branch; *c*, position of an Australian aboriginal near the same branch. Note another aboriginal at the base of the branch. Black circles, African ancestry; open squares, European; open triangles, Australian Aborigine; closed triangles, New Guinea; open circles, Asian.

the longer the vertical lines must be to separate them.

The effect of these distortions can be removed by redrawing the original data of Cann *et al.* so as to preserve the varying lengths of the branches and to avoid introducing any arbitrary separations between them (see figure). The result is an unrooted tree like a starburst, with the viewer looking back in time, and with the origin of the tree somewhere in the middle of the starburst. The tree shown is one of the many trees that are shorter than the original one, trees that can easily be generated by PAUP⁸, and the 'African' branch is near 12 o'clock. In this typical tree, the branch includes Asians, with one European and two Aborigines very close to it. When the data are looked at in this way, the impossibility of positioning the tree's origin with any certainty can easily be understood.

The starburst plot emphasizes the remarkable overdispersal of the data, with very unequal branch lengths and members of most groups scattered from the centre of the starburst to its periphery. These data are not alone in showing this overdispersed character, which has bedeviled the construction of phylogenetic trees from the beginning⁹. The impor-

tant question now becomes whether it is possible, by some combination of population subdivision, migration and fluctuating population sizes, to generate such a tree assuming a constant rate of neutral mutations, or whether it is necessary to postulate fluctuating mutation rates and/or selective pressures in the various branches.

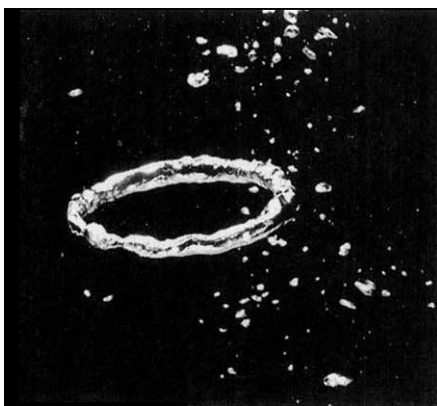
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Toroidal bubbles

SIR — The discussion about toroidal air bubbles by Daedalus¹ and Spisak² prompts me to mention our exhibit called "Friendship Acrobatic Troupe" by artist-in-residence Carl Cheng, in which a series of toroidal bubbles are formed in a 4-foot-deep transparent water tank through the actuation of a valved air jet. These toroidal bubbles float to the surface in an almost perfect ring. Smaller, detached bubbles clearly show the rota-



Toroidal air bubble.

tional flow of the liquid around the outside of the air ring. The toroidal rings are remarkably stable as they ascend up the tank (see figure). The bubbles are particularly beautiful because of the silver reflection produced by the light, which is incident directly from above and reflects due to total internal reflection.

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Alzheimer's — a correction

SIR — Lucotte and colleagues reported in Scientific Correspondence¹ the occurrence of a mutation (valine for isoleucine) in the β -amyloid precursor protein (APP) in a family in which both dementia and congophilic angiopathy occurs. G. A. and A. D. were responsible for the clinical assessment, DNA collection and neuropathological assessment of individuals from this family.

We have now carried out a further analysis of the APP gene. We used a *BclI* digest of the amplified product of exon 17 as a method for screening for the mutation². Despite the fact that we could detect the mutation in positive controls, *BclI* failed to digest any samples from this family. Therefore, we sequenced exons 16 and 17 of the amplified product, which revealed these exons to be of normal sequence in this family. Thus we regard the report by Lucotte and colleagues of the mutation in this family to be unreliable.

The valine to isoleucine mutation in exon 17 of the APP gene has been reported in six other families with Alzheimer's disease^{2–4}; in these families its presence has been confirmed by direct sequencing. Further, two other mutations in the same amino acid have been reported^{5,6}. Thus, we are confident that mutations to this amino acid can cause Alzheimer's disease. We recommend that the occurrence of all mutations should be confirmed by direct sequencing.

The range of the phenotype of the mutation in exon 17 remains to be precisely delineated. Clearly, however, the age of onset of the disease is a variable feature (about 48 to about 60 years) as is the occurrence of Lewy bodies². So far, no patients in which this mutation has been confirmed have more than modest congophilic angiopathy.

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Absorption of flue gases by water

SIR — Flue gas desulphurization by means of seawater scrubbing is an effective technique for reducing industrial emissions of sulphur dioxide (SO_2) with reported efficiencies as high as 99% (refs 1,2). Conventional scrubbers, however, require relatively large volumes of sea water to maintain the necessary liquid-to-gas ratios. We have carried out preliminary studies of SO_2 absorption in sea water to assess the potential of injecting flue gases directly into the sea.

A known volume of sea water (300 cm^3) was placed in a 500- cm^3 Dreschell bottle with a sintered frit. A stream of SO_2 in nitrogen (4,000 p.p.m.v.) was bubbled through the water at a set flow rate, and the concentration of SO_2 at the exit of the bubbler was monitored continuously using an infrared analyser (ADC model RF2A) and chart recorder. The pH of water was measured at the beginning and end of each run.

Typical absorption efficiency-time profiles are shown in Fig. 1. Initially all the SO_2 was absorbed, but after about 10 min the absorption efficiency began to fall off, reaching almost zero after 2 hours. Absorbed SO_2 is rapidly oxidized to sulphate in sea water³ resulting in acidification of the reacting solutions. It was possible to evaluate the SO_2 absorption capacity of sea water as $1.3 (\pm 0.1)$ g SO_2 l^{-1} from absorption curves in five experiments. Increasing the flow rate from 0.8 to 3 l min^{-1} had no significant effect on the absorption of SO_2 .

The final pH in all experiments was about 2, indicating that the decrease in absorption efficiency was due to the lowering in pH as a result of SO_2 absorption. That SO_2 solubility decreases with decreasing pH is well established², but sea water can accommodate significant quantities of acid without a significant change in pH. Bicarbonate was the main buffer. The buffer capacity was studied by adding increasing quantities of a stan-

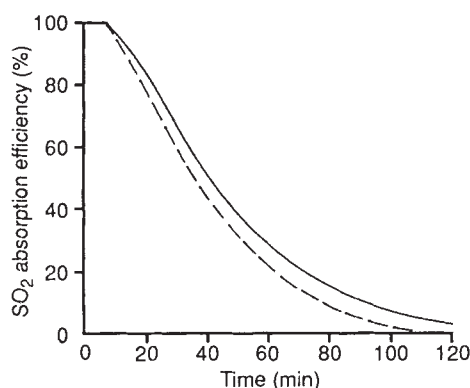


FIG. 1 Sulphur dioxide absorption efficiency in sea water versus time. Solid line, instant ocean sea water; dotted line, Mersey brine.

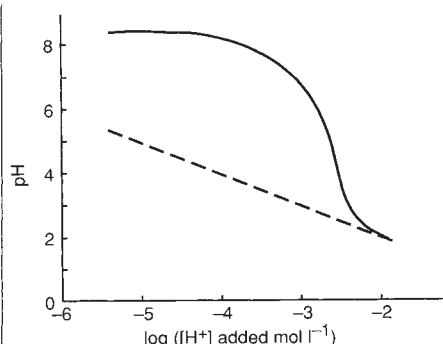


FIG. 2 Decrease in seawater pH as a function of added sulphuric acid. Similar curves were obtained with natural seawater samples. Solid line, instant ocean sea water; dotted line, pure water.

dard sulphuric acid solution and monitoring the resultant pH. A typical curve is shown in Fig. 2. The buffer capacity begins to break down at around 10^{-3} mol H^+ l^{-1} , and at 10^{-2} mol H^+ l^{-1} any advantage of using sea water over pure water is lost.

Sea water is a concentrated electrolyte solution containing high levels of chloride, sulphate and bicarbonate ions. Flue

gases from a 2,000-MW power plant emitting 4×10^3 g SO_2 s^{-1} and 2×10^3 g NO_x s^{-1} could be discharged directly into an ocean current with no perceptible effect on seawater pH or composition. Emissions of toxic metals associated with particulate matter may be minimized by passing the flue gases through suitable dust collection equipment, whereas NO_x species could be reduced by using NO_x abatement technologies, before piping the flue gases out to sea. In any case these would be diluted to background levels given the high transport rates of ocean currents (10^7 – 10^8 m^3 s^{-1}).

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Density-dependent populations

SIR — The apparently chaotic dynamics generated by a deterministic but non-linear relation between successive population densities have cast doubts on the efficacy of the standard methods of detecting population density-dependence¹. We believe that these doubts are unfounded.

We applied the conventional standard method of detecting density-dependence² to the deterministic relation between successive population densities $N_{t+1} = N_t \exp[r(1 - N_t/K)]$, where N_t denotes population density at time t , r is the intrinsic relative growth rate and K is the equilibrium density. Chaotic dynamics are generated by values of $r > 2.6294$. The analysis used 1,000 successive series of 10 values generated by the model with $K=1,000$ and for each $r=2.7, (0.1), 3.1$. The test was also applied to a stochastic version of the model in which N_{t+1} is assumed to be a Poisson variate with mean $N_t \exp[r(1 - N_t/K)]$. The results are presented in the table. Even for a series as short as 10 observations, density-dependence is detected in more than 99% of the cases at the 5% level.

Clearly, chaos induced in this instance by a first-order difference equation does not prevent the detection of density-dependence by the standard method. Similarly, in the case of chaos induced by second or higher-order difference equations it is confidently expected that the underlying signal will be detected by

NUMBER OF CASES IN WHICH BULMER'S TEST DETECTED DENSITY-DEPENDENCE

r	Deterministic model		Stochastic model	
	5%	1%	5%	1%
2.7	1,000	1,000	999	999
2.8	1,000	1,000	990	983
2.9	993	957	992	974
3.0	996	990	996	974
3.1	997	988	993	976

We used 1,000 sets of 10 observations at the 5% and 1% levels of significance in the model $N_{t+1} = N_t \exp[r(1 - N_t/1,000)]$.

the standard tests for stationarity of autoregressive schemes. Thus chaos does not in any way invalidate the conventional tests of detecting density-dependence.

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MAY REPLIES — My News and Views article¹ referred mainly to population models built up from assumptions about the behaviour of the constituent individuals (as distinct from those with phenomenological, population-level parameters, as above). I discussed how some kinds of noise in some behavioural parameters can, if nonlinearities in the

overall dynamics are strong enough to produce chaos, lead to situations in which conventional methods of data analysis fail to reveal the density-dependent mechanisms that are built into the model (and I cited an example drawn from experimental data³). Other kinds of noise in parameters characterizing the behaviour of individuals create no such problems. Why some situations should result in difficulties in detecting density dependence, while others do not, remains the subject of investigation.

Mountford and Rothery's observation that no problems arise in their simple, phenomenological model is interesting. But it does not really address the main point in the paper by Hassell *et al.*³ or my News and Views article¹.

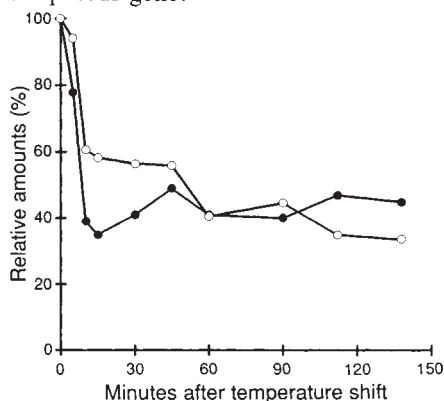
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Is yeast TCP1 a chaperonin?

SIR — G. North in his News and Views article¹ reports that the thermophilic bacterium *Sulfolobus shibatae* contains a gene, *TF55*, that is very similar in sequence to *TCP1* (ref. 2), suggestive of similar function. *TCP1* has a similar nucleotide sequence in mouse³, human³, hamster², *Drosophila melanogaster*⁴ and *Saccharomyces cerevisiae*⁵, making it a ubiquitous gene.



S. cerevisiae *TCP1* and actin transcript levels of wild-type strain M10 (ref. 5) after heat-shock. Relative transcript levels of *TCP1* (solid circles) and, for comparison, actin (open circles). Cultures were shifted at time 0 from 24 to 38 °C. The filter used to hybridize the *TCP1* probe was rehybridized with an *ACT1* probe. The absolute amounts of radiolabelled probe hybridized to specific bands was quantified on a Betascope Blot analyser (Betagen). One hundred per cent of counts for *TCP1* and actin represent 1,810 and 6,487 counts, respectively.

The data concerning the heat-shock properties of *TF55* in archaeobacteria do not extend to its homologues in yeast and *Drosophila*. In yeast, the opposite is true. Transcription of *TCP1* is repressed during heat stress as shown in the figure. Within 10 min after temperature shift from 24 to 38 °C the *TCP1* transcript level decreases by about 60%, and remains low thereafter. The upstream non-coding sequence of yeast *TCP1* does not contain the consensus elements required for heat-induced transcription⁶. In agreement with the findings in yeast, the *D. melanogaster* homologue⁴, which maps to the chromosomal location 94B, is not associated with either the major or minor heat-shock puffs⁶ and thus is not in a chromosomal region known to encode a heat-inducible protein.

Another important characteristic of most chaperonins, that they are members of a multigene family, is not shared by the *Drosophila* or yeast *TCP1* genes. The *TCP1* genes in both organisms have no detectable sequence homologues and thus do not appear to be members of a family of related genes. If yeast and *Drosophila* *TCP1* are indeed cytoplasmic chaperonins, they do not share two important attributes found to be present in most chaperonins. This would make *TCP1* a remarkable chaperonin.

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Crystallization by centrifugation

SIR — The Scientific Correspondence by J. E. Pitts¹ about the crystallization of a protein by centrifugation is not unprecedented. First beef liver catalase and later fungal (*Penicillium vitale*) catalase were crystallized by ultracentrifugation and reported in the Soviet journal *Kristallografiya*^{2,3}. The work was later published in English in *Soviet Physics, Crystallography*^{4,5}. V. Barynin presented this work at the FEBS advanced lecture course dedicated to the crystal growth of biological macromolecules (Bischenberg, Alsace, 19–25 July, 1987)⁶.

The Russian authors mentioned that the first virus coat protein was crystallized

in an ultracentrifuge as early as 1936⁷. Ironically, a model of the three-dimensional structure of *P. vitale* catalase, determined using the crystals grown by ultracentrifugation⁸, was built in the early 1980s on the graphics system at Birkbeck College, London.

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PITTS REPLIES — Abad-Zapatero correctly refers to the production of catalase and tobacco mosaic virus (TMV) protein crystals in an ultracentrifuge^{7,8}. In my Scientific Correspondence I alluded to this effect when I indicated that “high g-force” had been used successfully to approach the problem of crystallization. But apart from being crystallized while rotating in a centrifuge, the two methodologies are fundamentally distinct. The catalase crystals were grown at 26,000g in a high-speed ultracentrifuge over 180 hours (7½ days) in the presence of a precipitant 2-methyl-2,4-pentenediol (MPD), while the large particle size TMV crystals are produced at 40,000g^{7,8}.

The crystals of the aspartic proteinase from *Trichoderma reesei* are grown rapidly (2½ hours), at low speed and ionic strength, during rapid concentration through an ultrafiltration membrane at quite low (3,000g) centrifugal force. The nearest comparable method reported in the literature would be that of concentration dialysis⁹. The concentration process to produce protein crystals being achieved either by pressure dialysis under nitrogen¹⁰ or by vacuum dialysis through conical collodian membranes^{11,12}. The commercial availability of centric concentrators (Amicon) and of suitable slow-speed centrifuges with a fixed-angle rotor in most laboratories involved in protein purification should mean that the technique could be used more generally.

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HIV infection in Africa

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AIDS in Africa. Edited by Peter Piot, Bila Kapita and JBO Were. *Current Science*: 1991. Pp. 209. £35, \$59 (pbk).

Aids in Africa: Its Present and Future Impact. By Tony Barnett and Piers Blaikie. *Belhaven*: 1992. Pp. 193. £35 (hbk), £9.99 (pbk).

THE effects of infection with the human immunodeficiency virus (HIV) were first recognized in homosexual men in North America and Europe; 10 years later the developing world faces a massive epidemic of heterosexually spread HIV infection, the effects of which will not become fully apparent for some years. Current estimates suggest that 60 per cent of those infected with HIV may live in Africa, where in some areas infection has already reached appallingly high levels; in these places AIDS is killing large numbers of people in their most productive years and in some hospitals it may be the most common cause of death among adults in medical wards. Yet comparatively few of the resources of the international scientific community working on HIV have been channelled into Africa. This is shown by the recent finding that less than 4 per cent of the papers on HIV in biomedical journals would seem to have discussed the problem of AIDS in Africa. These two complementary publications are therefore most welcome, providing up-to-date accounts of what is known about the epidemiology, effects and possible control of HIV infection in Africa; together, they document the enormous problems posed by the epidemic.

Assessing the impacts

AIDS in Africa, edited by Piot, Kapita and Were, is a special supplement to the journal *AIDS*. The results of the research that has been done in Africa are scattered widely throughout the scientific literature and much has appeared only in the form of conference abstracts. The 26 authoritative review articles in the supplement are a valuable summary of recent thinking about most aspects of the problems posed by HIV infection, with some emphasis on issues that are of interest to clinicians and epidemiologists. In his foreword, Michael Merson, director of the World Health Organization's global programme on AIDS, comments on the difficulty of visualizing in human terms the meaning of the dry epidemiological statistics that describe the African epidemic. But this task should be easier for readers of *Aids in Africa: Its Present and Future Impact* by Barnett and Blackie, which is more limited in scope and concentrates on the social, demographic and economic

consequences of the infection in one of the worst-affected areas of Uganda, although wider issues are also considered. The authors' account of 18 months of fieldwork in the Rakai district includes detailed descriptions of the complex and tragic ways in which morbidity and mortality due to HIV infection have affected individual families.

Both volumes contain discussions of some of the successful examples of patient care, counselling and prevention programmes. Unfortunately, these are relatively isolated achievements. Most Africans affected by HIV remain without appropriate treatment for opportunistic infections, with only a few of the elite having any access to antiretroviral therapy. The resources for programmes to persuade people to change their sexual behaviour or to encourage them to use condoms do not appear to match those devoted to the marketing of soft drinks or cigarettes. In their review

article, Peter Lamprey and Gail A. W. Goodridge acknowledge that in most parts of Africa condoms are either not available or not being used by those who need them.

Barnett and Blaikie realistically point out that the acquisition of knowledge does not necessarily lead to a change in behaviour. They discuss in detail some of the ways in which health education in Uganda may be absorbed into a syncretic view of causality; this can include ideas from traditional beliefs about witchcraft and a fatalistic view of life that allows a rationalization of continued high-risk behaviour. They also consider the problems facing many women in Africa whose position may make it difficult, or impossible, to discuss the use of condoms with their partner or to share the results of an HIV test. It thus is no surprise that in a review of prevention and control strategies, David L. Heymann and Karin Edström anticipate a considerable increase in HIV infection among African adults during the 1990s.

Prospects of a vaccine

Merson mentions the ray of hope given by the prospect of a vaccine, and it is encouraging to know that the World Health Organization is considering Uganda and Rwanda as sites for vaccine trials. But the development of a vaccine that can be used widely in Africa will



'HANDY-MAN' — The famous skull ER1470 of *Homo habilis* (Upper Pliocene, about 1.9 million years old) discovered by Richard Leakey in East Turkana, Kenya. This is one of 160 superb colour plates reproduced in *Fossils: The Evolution and Extinction of Species* by Niles Eldredge, which covers more than 250 different fossil specimens, most taken from the American Museum of Natural History. Published by Aurum, London/Abrams, New York. Price £29.95, \$60.

need a considerable expansion of studies of African HIV isolates. In a review of variation in African HIV strains, Martine Peeters, Piot and Guido van der Groen discuss the need for further studies of the genetic and antigenic variation of HIV in different geographical regions. Vaccines will have to be effective against an array of isolates and it will be necessary to define the importance of genetic differences in relation to functional immune responses. West Africa also poses the problem of the HIV-2 virus; although there is increasing evidence that this is less pathogenic and less readily transmitted than HIV-1, the terminal stages of the infection are similar to those seen with HIV-1.

The review articles in the supplement to *AIDS* highlight many other issues for the research agenda. But although agreeing that medical research is a priority, Barnett and Blaikie emphasize that this must be supplemented by studies of the economic and social impact of the

epidemic and of the way in which affected African communities cope with its effects.

Writers in both volumes recognize that continued international support for control programmes and research on HIV in Africa is threatened; among the factors involved are an increasing complacency about the effects of the infection, a growing lack of interest in the problems of development in Africa, competition from other programmes and the absence of obvious, spectacular successes in AIDS control. Although the perspective and aims of these two volumes differ, readers of either will be convinced of the magnitude of the present and probable future effects of HIV infection in the continent and of the need for the wider international community to be more supportive of Africa's response to the pandemic. □

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Unpredictable reactions

John J. Tyson

Chemical Chaos. By Stephen K. Scott. Oxford University Press: 1991. Pp. 454. £60, \$135.

CHAOS is a common part of everyday life. My desktop, toolbox and sock drawers are typically in a chaotic state. This type of chaos goes by the technical name 'entropy', which is very law-abiding, predictable stuff. But there is another type of randomness ('deterministic chaos') whose defining feature is not so much disarray as unpredictability. Deterministic chaos refers to a recurrent process that never exactly repeats itself. Furthermore, even though the process is governed by precise mathematical rules (such as some differential equations), its long-term evolution is irreproducible: two initial states, no matter how similar, will lead eventually to uncorrelated outcomes. Therefore, because the initial state of a real system can never be known with infinite precision, the long-term behaviour of a real chaotic system is fundamentally unpredictable.

That deterministic systems can behave unpredictably and irreproducibly is a blow to the scientific ethos to say the least. These revolutionary ideas first surfaced clearly in a toy mathematical model of fluid flow, in a theoretical study of biological population densities, and in experimental observations of some obscure chemical reactions.

Chemical chaos manifests itself as an

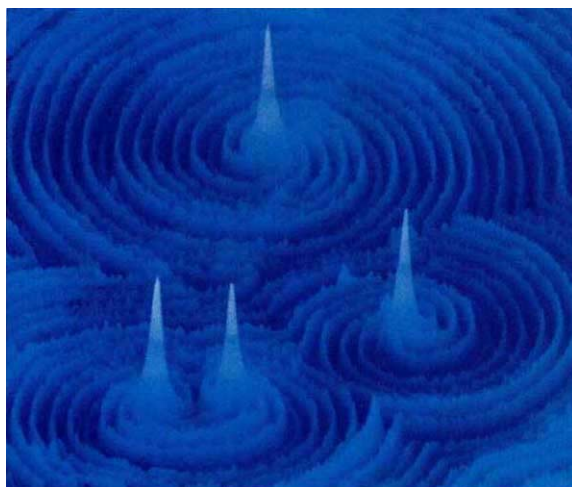
irregular oscillation in some indicator of the progress of a chemical reaction, for instance a fluctuating electrode potential. The shining example of chemical chaos is the venerable Belousov-Zhabotinskii reaction (the oxidation of malonic acid by bromate, catalysed by iron ions) operating in a continuously stirred flow reactor at certain precise values of the flow rate. Irregular oscillations are also observed in gas-phase reactions (the oscillatory combustion of hydrogen and oxygen mixtures), in heterogeneous processes (the oxidation of carbon monoxide on platinum crystals or the dissolution of copper electrodes) and in biochemical systems (the horseradish peroxidase reaction).

But what exactly is an 'irregular oscillation'? Is it a perfectly periodic oscillation that is randomly perturbed by

the stirring and pumping machinery of the reactor, or could it be an almost-periodic oscillation similarly perturbed, or perhaps a complex concatenation of oscillatory states that would be perfectly periodic if only our experiments were accurate enough? Interesting as they may be, none of these possibilities is deterministic chaos as strictly defined. The fundamental challenge to proponents of chemical chaos is to prove that the irregular oscillations they observe are reflections of deterministic chaos in the underlying chemical reaction rather than random noise superimposed on a sensitive, but otherwise regular, chemical oscillator. This problem has bedevilled the field from the beginning and is still not totally resolved.

Scott's book is a serious and comprehensive introduction to chemical chaos. In language familiar to chemists, he explains all the essential theoretical ideas and important experimental examples. He wisely avoids technical details; readers who need to know how to calculate fractal dimensions or Lyapunov exponents can consult the literature cited. The book is a sequel to *Chemical Oscillations and Instabilities*, which he wrote with Peter Gray (Oxford University Press, 1990). Although the new volume can be read profitably on its own, the earlier volume presents a thorough overview of the exotic behaviour of nonlinear chemical reactions operating far from equilibrium: limit-cycle oscillations, multiple steady states and travelling waves, in addition to chaos. Serious students will want to own and study both volumes.

In *Chemical Chaos*, Scott reviews a broad selection of research articles and provides thorough reference lists. These services are useful to beginners trying to grasp the burgeoning literature on chemical chaos. Unfortunately, he does not give enough attention to some



THE Belousov-Zhabotinskii reaction. The catalyst is usually a mixture of cerium (II) and (III) ions and ferroin, which acts as a visual indicator. The plate shows a typical reaction oscillating in composition and thus colour. The reaction is discussed in a chapter by Stephen Scott in *The New Scientist Guide to Chaos* edited by Nina Hall. Other contributors include Ian Stewart, Robert May and Benoit Mandelbrot. Published by Penguin, £9.99 (pbk).

of the fundamental problems of the field: which observations are polluted by instrumental noise, and which are genuine deterministic chaos? And how do we know?

Despite my disappointment on this score, I can enthusiastically recommend the book to chemists who want to learn what all the fuss over chemical chaos is about. It is a no-nonsense book, written by a chemist for chemists, without unnecessary mathematical frills or philosophical fancies. The book contains not one colour figure, which I take as a good sign: it is meant not for the coffee table but for serious study by the next generation of chemical kineticists. □

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Mesmer again?

Cyril W Smith

Biomagnetism. By Romuald S. Wadas. *Ellis Horwood*: 1991. Pp. 170. £40.00, \$68.

IN 1987, the New York State Power Lines Project realized that "the . . . variety of effects of magnetic fields have not been previously appreciated". The whole area of biomagnetism continues to be fraught with controversy and partisan influence. As Romuald Wadas remarks:

Thousands of experiments have been carried out to date, and different conclusions reached therefrom, sometimes contradicting each other. A physical explanation of these effects encountered enormous difficulties. Until recent years, the possibility of a magnetic field having an influence on chemical reactions was altogether denied . . . The existence of such effects was looked upon as pure fantasy.

This book is a revised and enlarged translation from the Polish edition, *Biomagnetyzm*, published in 1978. The translation editor, Professor R. Pethig of University College of North Wales has ensured a well-prepared English text. The book is addressed to all those interested in the physics of magnetic phenomena in biology and claims to be the first book on biomagnetism available in English. It covers the fundamentals of magnetic phenomena together with magnetic molecular complexes, diamagnetic, paramagnetic and ferromagnetic biological molecules, magneto-optical phenomena and the effects of magnetic fields on living organisms through results of selected biological experiments.

What Wadas does well is to cover the physics of magnetic phenomena in a way more likely to be understood by the medical and biological reader, and to spell

out the implicit biochemistry for the benefit of the physicist. He explains that

[in] the course of a chemical reaction, when a molecule breaks down but its components have not yet had time to produce new diamagnetic molecules, there exist energy conditions for spin reversal by a magnetic field . . . The arrangement of molecule components passes from a singlet-excited to a triplet-excited state.

Thus can magnetic fields, including those generated by paramagnetic molecules and free radicals (paramagnetic on account of their uncompensated spins) and even nuclear magnetic moments, affect the course of chemical reactions.

Wadas describes how liquid crystal molecules, widespread in biological systems, can be structurally deformed by electric and magnetic fields to produce changes in optical activity and membrane properties. He gives a simplified classical description of magnetic resonance and explains what can be derived from measurements of resonance lines. He notes that with a stationary magnetic field of the order of 100 milliteslas in strength, there can be strong influences, both adverse and favourable, on the activity of biological molecules as well as on the blood system, cellular membranes, the operation of the heart, neoplastic cells, enzyme activity, and on the germination and development of plants.

The effects of Earth-strength (50 microteslas) magnetic fields are considered in relation to magnetic compasses in living organisms and radiaesthesia. Wadas quotes from Y. Rocard's chapter in M. F. Barnothy's book (*Biological Effects of Magnetic Fields*, Plenum, 1964) on the biological effects of magnetic fields, mentioning the threshold of 30 nanoteslas per second for dowsing a magnetic-field gradient and the 2–5-microtesla saturation level, 'windows' that may represent bands within which some form of long-range coherence is established.

Four-fifths of the references date from the 1978 Polish edition, which further restricts the value of a book already priced beyond the reach of most graduate students. The substantial literature following the publication of P. Semm, T. Schneide and L. Vollrath's report in *Nature* on "Effects of an Earth-strength magnetic field on electrical activity of pineal cells" (288, 607; 1980) has not been noted; neither has the work of H. Fröhlich on coherence in living systems.

Judging by the programme list for a forthcoming international congress, the book is a decade too late to make an impact among researchers in biomagnetics, but still a decade too early for others to appreciate the significance of the subject. □

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The Universe defined

R. J. Tayler

The Astronomy and Astrophysics Encyclopedia. Edited by Stephen P. Maran. *Cambridge University Press*: 1992. Pp. 1002. £60.

A Concise Dictionary of Astronomy. By Jacqueline Mitton. *Oxford University Press*: 1991. Pp. 423. £12.95, \$19.95.

THE briefest of perusals of these two reference books suffices to show just how dramatically our knowledge of the Universe has increased in the past 50 years. In 1940, observational astronomy meant optical astronomy, whereas now the Universe is studied in all regions of the electromagnetic spectrum. Larger optical telescopes and more efficient detectors, many carried by satellites, have allowed astronomers to probe deeper into space and hence further back in time. Many new types of objects, such as pulsars, binary X-ray sources, infrared protostars, quasars and radio galaxies, are now known; and, through the discovery of the cosmic microwave radiation, the study of the very early Universe has been made respectable. Even the Solar System is seen in a different light. Space missions have provided detailed information about the atmospheres and surfaces of the planets, led to the discovery of new satellites and allowed detailed measurements of the properties of the Earth's local environment.

The Universe now provides a physical laboratory in which the laws of physics can be tested in a way that is not possible on Earth. White dwarfs once provided a test of quantum mechanical predictions of the properties of a dense degenerate electron gas. Now neutron stars give information about bulk matter at and above nuclear densities; Einstein's general theory of relativity has been given strong support by the discovery of gravitationally lensed quasars and the properties of the binary pulsar; and plasma physics and magnetohydrodynamics are new branches of physics that can be studied particularly well in the interaction of the solar wind and Earth's magnetosphere. Particle physicists have realized that they can never build accelerators to compete with conditions in the very early Universe, and the union of particle physics and cosmology is one of the active fields of current research. But particle physicists in a sense have had their revenge, for it now seems that most of the matter in the Universe is nonluminous, consisting of weakly interacting elementary particles rather than ordinary matter. Even chemistry

has benefited from a two-way interaction between studies in the laboratory and the discovery of complex interstellar molecules.

Most of the entries in *The Astronomy and Astrophysics Encyclopedia* could not have been written in 1940, and many of the others would have been totally different. Commissioning such an encyclopaedia is an ambitious undertaking, and Stephen Maran and his editorial advisory board and contributors must be congratulated on the successful manner in which they have carried out this task. Although it would be an exaggeration to say that the whole of present astronomical knowledge is contained in these densely packed pages, most active research fields are summarized and references are given to enable readers to get more deeply into a subject. Cross-referencing between articles is extensive; most of them were written in 1989 and 1990, with many brought up to date while in proof in 1991. The encyclopaedia thus presents a broad picture of present-day astronomical knowledge and research.

I have not read every word of the encyclopaedia, nor do I have the knowledge to confirm that all that I have read is sound. I am however happy that most of the articles are clearly written and provide a realistic and up-to-date view. Some currently unfashionable views are perhaps underrepresented and could have surely been mentioned without giving them strong support. I think, for example, of active galactic nuclei without black holes and of the capture theory of the origin of the Solar System.

Jacqueline Mitton's *A Concise Dictionary of Astronomy* should be extremely useful to anyone who wishes to find a short definition of a word or phrase relating to astronomy. She too has made use of extensive cross-referencing between entries, ensuring that no text has to be repeated. She has also wisely decided not to include any astronomers by name. It would have been an invidious task to decide who should be included and who should be left out, and one that would have lengthened the dictionary considerably. So astronomers appear only in conjunction with concepts named after them, such as Einstein ring, Eddington limit, Hertzsprung–Russell diagram, Hubble constant, Jeans mass and Oort cloud. I tested the range of contents of the dictionary by looking for entries that I would expect to find; only rarely was I disappointed, either by the absence of an entry or by an overstatement or inaccuracy. To show that I have read this highly recommended book, I will list just a few suggested entries for the second edition: asymptotic giant branch, giant branch, interstellar dust, Oort limit, pre-main-sequence star.

It is not easy to envisage what these two books will look like in 2040, but I am sorry that I shall not be around to see them or the transformation in understanding that will have occurred. □

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An insider's view

David J. Stevenson

Deep Interior of the Earth. By J. A. Jacobs. Chapman and Hall: 1991. Pp. 167. £24.95, \$39.95.

It is no accident that Earth scientists devote so much attention to the parts of our planet that are nearby. The data are voluminous, the supporting experiments are easier and the issues are well-defined, pressing and still often unresolved. The deep interior, and especially the core, has usually been most attractive to those who like their science unencumbered by too many awkward facts or richness of detail. But this area of research is maturing and now offers much more substance to go along with the ever-present excitement of dealing with basic questions such as why the Earth has a large magnetic field. Four areas of endeavour are principal contributors to this flourishing activity: seismology, geomagnetism, mineral physics and fluid dynamical convection modelling. A particularly important development of recent years has been the growing acceptance and understanding of how the Earth's core and mantle interact chemically, thermally and dynamically. It is no longer appropriate to focus on one region without regard to the other.

Jack Jacobs' new book is a modest but successful summary of these areas of study. It is up to date, with many references as recent as 1991, and covers all the important subjects in a largely non-mathematical but substantive way. Jacobs' style has been honed in several books, including a much longer study of the Earth's core, and consists of reporting the science rather than developing a personalized synthesis. This has both advantages and disadvantages. The advantage is that the reader is presented with a usually thorough and unbiased summary of the state of affairs, something that is not guaranteed even in a thoughtful review paper where a writer is prone to emphasizing his or her own efforts. The disadvantage is that the reader cannot really judge the work because Jacobs frequently eschews judgement or even criticism in favour of

straight reportage. My feeling is that a thoughtful, provocative synthesis can have a far greater and lasting impact on the field, but that a book such as this also serves an important role as a source of information.

Readers of Jacobs' earlier books should be assured that his latest is not merely an update but covers some new ground, including considerable discussion of the lowermost mantle and the nature of mantle convection. The most suitable readers, however, are not those already aware of Jacobs, but a much broader set of scientists, those who do not research the deep Earth but who wish to appreciate the current state of our understanding. For this, the book is extremely appropriate because it is clearly written, succinct and well illustrated (mainly by figures extracted from published papers). Although it is not suitable as a class textbook, it is an excellent supporting sourcebook and I have already used it in this way in a survey geophysics course. It even includes a short but thorough summary of ideas about the Earth's origin, which is most appropriate bearing in mind that the nature of the core is probably closely connected to how it formed. □

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New in paperback

■ Icon Books have recently relaunched four bestselling cartoon guides to contemporary issues: *Freud for Beginners* by R. Appignanesi and O. Zarate; *Ecology for Beginners* by S. Croall and W. Rankin; *Einstein for Beginners* by J. Schwartz and M. McGuinness; and *Darwin for Beginners* by J. Miller and B. van Loon (for a review see *Nature* **300**, 551; 1982). Each is priced £7.99.

■ *The Legacy of Chernobyl* by Zhores Medvedev is a full and accessible account of the disaster. Published by Norton, price \$10.95. For a review see *Nature* **352**, 283; 1991.

■ In *Blinded by the Light: The Secret History of the Sun*, John Gribbin provides an engaging summary of the latest research on the Sun. Published by Black Swan, price £5.99. For a review see *Nature* **355**, 29; 1992.

■ Stephen Jay Gould's most recent collection of essays, *Bully for Brontosaurus*, is issued in paperback by Penguin, price £7.99. For a review see *Nature* **352**, 117; 1991.

■ John Barrow's *Theories of Everything: The Quest for Ultimate Explanation* is published in paperback by Vintage, price £6.99. For a review see *Nature* **350**, 325; 1991.

■ Cambridge University Press reissue next month the classic *A Mathematician's Apology* by G. H. Hardy, with a forward by C. P. Snow. Price £4.99, \$7.95.

■ Andrew Hodges' biography of Alan Turing, *The Enigma*, is reissued by Vintage with a new preface. Price £8.99. For a review see *Nature* **307**, 663; 1984.

Social controls on cell survival and cell death

Martin C. Raff

Programmed cell death occurs in most animal tissues at some stage of their development, but the molecular mechanism by which it is executed is unknown. For some mammalian cells, programmed death seems to occur by default unless suppressed by signals from other cells. Such dependence on specific survival signals provides a simple way to eliminate misplaced cells, for regulating cell numbers and, perhaps, for selecting the fittest cells. But how general is this dependence on survival signals?

AFTER many years of neglect, normal (or programmed) cell death is at last coming alive. It has long been known to be a fundamental feature of animal development¹⁻³ and to occur in many adult tissues⁴⁻⁶, but only very recently has it become a fashionable subject of general biological interest. Although the molecular mechanisms are unknown, it is thought that the cells that die usually kill themselves by activating a suicide programme⁷⁻¹⁰. Many studies of normal cell death have focused on what seem to be special cases: cells dying at metamorphosis, neurons dying during synaptogenesis, lymphocytes dying during receptor repertoire selection, and so on, and cell suicide has not been thought to be a part of the repertoire of cells in general. But there is increasing evidence to suggest that most animal cells are capable of killing themselves and that these ubiquitous cell suicide programmes can be activated or suppressed by signals from other cells¹⁰. It seems that cell survival and cell death are subject to the same kinds of social controls that operate on cell proliferation; yet compared with the control of cell proliferation, relatively little is known about the control of cell survival.

Cell suicide provides an efficient mechanism for eliminating unwanted cells⁸. These are usually normal cells that are unwanted for any one of a number of reasons⁹. They may have been produced in excess, as is the case for many vertebrate neurons¹¹⁻¹³. They may have served a function at some time during evolution or development but are no longer needed, as in the tadpole tail at metamorphosis^{14,15}. Some cells are needed in one sex but not in the other, as in the mammalian Müllerian duct, which is needed in females but not males¹⁶. Others have to be sacrificed in the process of sculpting the body^{1,2}, as in the regions between developing digits in amniotes¹⁷. Cells that migrate to an abnormal location may be eliminated by cell suicide, as may lymphocytes that have either failed to produce functional antigen-specific receptors and therefore are of no use, or have produced high-affinity self-reactive receptors and therefore are potentially harmful to the animal^{18,19}. In each of these cases, it is easy to see the advantage of a cell suicide programme that can be regulated by interactions with other cells.

Cell suicide by default

An extreme view is that, in higher animals at least, just as a cell seems to need signals from other cells in order to proliferate²⁰⁻²², so it needs signals from other cells in order to survive; in their absence, the cell kills itself by activating an intrinsic suicide programme. The view is extreme because it implies that cells are programmed to kill themselves unless they are continuously signalled by other cells not to do so, and because it suggests that this precarious state is shared by most cells in both developing and mature higher animals.

Evidence for cell suicide

The evidence that normal cell deaths occur by suicide is indirect. In some cases, an increase in the synthesis of specific messenger RNAs has been shown to precede the cell deaths²³⁻²⁶, although it has yet to be shown that any of the proteins encoded by these mRNAs contributes to cell death. Death can sometimes be

suppressed by the inhibition of RNA or protein synthesis in the cells that should die²⁷⁻²⁹, suggesting that gene transcription and RNA translation are required in these cells for the deaths to occur.

As originally pointed out by Kerr, Wyllie and Currie³⁰, the cytological characteristics of normal cell deaths are generally different from those seen in acute pathological cell deaths. In acute pathological cell deaths, which result from cell injury, the cells tend to swell and lyse—a process called necrosis^{7,30,31}—and the cell's contents spill into the extracellular space, inducing an inflammatory response. In normal cell deaths, by contrast, the nucleus and cytoplasm shrink and often fragment, and the cells or fragments are rapidly phagocytosed by their neighbours or by macrophages—a process called apoptosis^{7,30,31} (Fig. 1); as the contents of the cell do not leak into the extracellular space, there is no inflammation. Because the dead cells are rapidly removed without inflammation, even large-scale normal cell death is often histologically inconspicuous and therefore ignored, which is one important reason why it has received less attention than it deserves. Normal cell deaths can vary in their features, however, even in the same organism^{9,32,33}; in some cases, for example, the nuclear DNA is degraded into oligomers of oligonucleosome-sized fragments^{8,33-35}, whereas in others it apparently is not³³. It is uncertain whether the variability reflects differences in the mechanism of cell death, although this seems likely³³.

The most direct evidence that normal cell death in animals is caused by the activation of a suicide programme comes from studies in the nematode *Caenorhabditis elegans*⁹. Of the 1,090 somatic cells formed during the development of an adult hermaphrodite, 131 die, each with morphological features resembling apoptosis⁹. Genetic analyses have identified two genes, *ced-3* and *ced-4* (ref. 36), that must function in the dying cells or their close ancestors for these cells to die³⁷; if either gene is inactivated by mutation, the deaths fail to occur. These mutant worms are superficially normal³⁶ and die at the usual age of several weeks⁹, indicating that although the cell deaths that occur in normal development involve a *ced-3*- and *ced-4*-dependent death programme, senescence does not. The DNA sequences of both genes have been determined; *ced-3* encodes a novel protein with many potential phosphorylation sites, whereas *ced-4* encodes a novel protein with two potential Ca²⁺-binding domains⁹, but it is not known how either protein participates in cell death.

From the perspective of the extreme view, another *C. elegans* gene, *ced-9*, is of special interest as it seems to act as a brake on the suicide programme³⁸. If its function is abnormally activated by mutation, the cell deaths do not occur; if its function is inactivated by mutation, many cells that normally survive now die and the animal dies early in development. If *ced-3* and *ced-9* are both inactivated, neither the normal nor extra cell deaths occur and the animal survives³⁸. These results suggest that many more cells than the 131 that normally die during nematode development have the *ced-3* and *ced-4*-dependent suicide programme ready to run, but it is normally suppressed in these cells by *ced-9*-dependent mechanism. The extreme view that

cells need continuous signalling from other cells to avoid killing themselves would gain support if the activity of *ced-9* were found to depend on signals from other cells, but this possibility has not been tested. In one respect the mammalian gene *bcl-2* resembles *ced-9*; when overexpressed in some cells, such as lymphocytes, it can protect them from programmed cell death³⁹⁻⁴¹; in the case of B lymphocytes isolated from germinal centres, both the expression of *bcl-2* and cell survival are stimulated by ligand binding to antigen-specific receptors on these cells⁴². The finding that the BCL-2 protein is mainly localized in the inner mitochondrial membrane⁴³ raises the possibility that mitochondria may play an important part in at least some forms of programmed cell death.

The death programme in animal cells can clearly be activated by signals from other cells. Cells in the tadpole tail, for example, are induced to die by thyroid hormone secreted by the thyroid gland at metamorphosis^{27,44}, and lymphocytes in the mammalian thymus are induced to die by corticosteroid hormones secreted by the adrenal gland¹⁸; in both cases, the deaths have the characteristic features of programmed cell death, in that they occur by apoptosis^{14,35} and can be suppressed by inhibitors of RNA or protein synthesis^{27,45}. Both cytotoxic lymphocytes and tumour necrosis factor, which is secreted mainly by lymphocytes and macrophages, are able to stimulate many cell types to undergo apoptosis, although these deaths are not suppressed by RNA or protein synthesis inhibitors^{18,19}. Interestingly, the binding of antibodies to either the Apo-1 (ref. 46) or the Fas (ref. 47) protein on the surface of lymphocytes or other cells can also induce programmed cell death, and mutations in the gene encoding the Fas protein are associated with a defect in the elimination of self-reactive T lymphocytes in the thymus and, consequently, with autoimmune disease⁴⁸. More important for the extreme view, however, is the evidence that the survival of some cells depends on the continuous suppression of the death programme by signals produced by other cells.

Dependence on survival signals

Although the notion that cells in higher animals depend on signals from other cells to avoid killing themselves is extreme, at least some cells seem to operate in this way. The survival of many developing vertebrate neurons depends on neurotrophic factors that are secreted by the target cells they innervate^{11-13,49,50}; those that fail to get enough die, apparently by active suicide as, in some cases at least, their death can be prevented for days by drugs that inhibit RNA or protein synthesis²⁹. Dependence on signals from other cells for survival is not confined to neurons or to developing cells: in adult rats, for example, the survival of epithelial cells in the ventral prostate depends on testosterone secreted by the testes^{51,52}, whereas the survival of cells in the adrenal cortex depends on adrenocorticotrophic hormone (ACTH) secreted by the pituitary^{5,7}; if testosterone or ACTH levels are experimentally decreased, the respective cells die with the characteristic features of programmed cell death^{5,7,51,52}.

Among non-neural tissues, it is not only endocrine-dependent cells that require specific signalling molecules to survive: in culture at least, haemopoietic progenitor cells require one or more colony-stimulating factors⁵³⁻⁵⁵, T lymphoblasts require interleukin-2 (ref. 28), and endothelial cells require growth factors such as fibroblast growth factor⁵⁶; in all of these cases, cells deprived of survival signals die by typical programmed cell death.

Thus the idea that cells require signals from other cells to avoid killing themselves is not new. What is novel is the suggestion that these cases are only examples of a general mechanism that may operate in most cells, at least in higher animals. The simplest way to test whether a cell depends on survival signals produced by other cells is to determine whether it can survive on its own in culture without added signalling molecules. It is important to test highly purified cells in order to avoid signalling between cell types: studying single cells is the ultimate test as it also excludes signalling between cells of the same type. It will be important to determine which types of normal animal cells, other than blastomeres⁵⁷, can survive under these conditions. Chondrocytes from embryonic chick cartilage have been shown to survive in serum-free cultures at low density without added signalling molecules, but single cells were not tested⁵⁸. My colleagues and I recently tested newly formed oligodendrocytes (the cells that make myelin in the vertebrate central nervous system) and their precursors, both as purified populations and as single cells, isolated from the rat optic nerve⁵⁹. We found that they died rapidly, with the characteristics of programmed cell death, when cultured in the absence of serum and exogenous signalling molecules. They could be saved for at least a few days by molecules secreted in culture by their normal neighbours isolated from the developing optic nerve, or by recombinant platelet-derived growth factor or insulin-like growth factor-1, both of which are present in the developing optic nerve.

Tissue-specific survival signals

What might be the advantages of having a cell's survival depend on signals produced by other cells? One is that it could provide a simple mechanism for eliminating cells that end up in an abnormal location: as different vertebrate tissues would be expected to produce different sets of survival signals, a misplaced cell may be deprived of the specific signals required for its survival. Nerve cells (or their processes⁶⁰) that project to an abnormal target that cannot provide the necessary neurotrophic factors, for example, will be automatically eliminated¹². Primordial germ cells may be another example. They migrate early in vertebrate development from the hindgut to the genital ridges, where they ultimately differentiate into gametes⁶¹. Some fail to reach the genital ridges and are eventually eliminated⁶². It has been suggested that at least some of these misplaced cells in mice might die because they are deprived of an extracellular signalling protein encoded by the *Steel* (*Sl*) gene, which may be required for germ-cell survival⁶³. Several lines of evidence

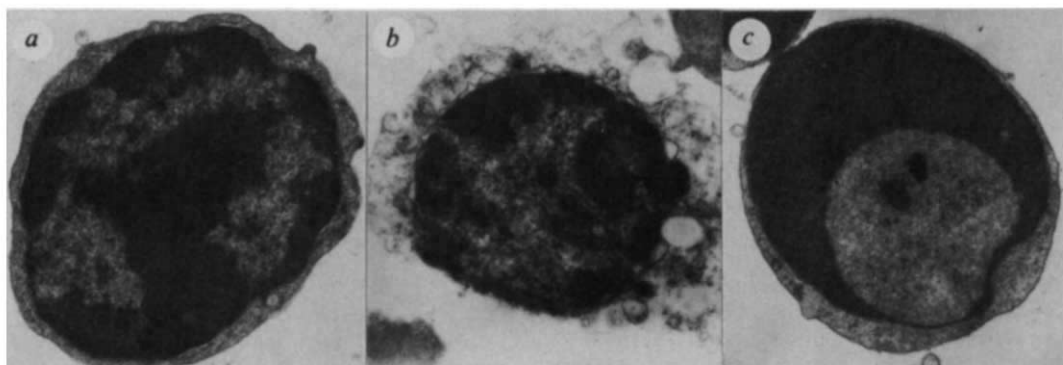


FIG. 1 Electron micrographs of a normal thymocyte (a), a necrotic thymocyte (b) that died after exposure to polyethylene glycol, and an apoptotic thymocyte (c) that died after exposure to glucocorticoid. Magnifications are $\times 15,000$ in a, $\times 12,000$ in b, and $\times 24,000$ in c. (Kindly provided by A. Wyllie from *J. Path.* **142**, 67-77, 1984.)

are consistent with this suggestion: (1) if either the *Sl* gene, which encodes the signal, or the *c-kit* gene, which encodes its receptor, is inactivated by mutation, the mouse is deficient in germ cells (as well as in erythrocytes and melanocytes)^{64,65}; (2) the *Sl* gene is expressed in the genital ridges and along the migratory pathway followed by primordial germ cells^{66,67}, whereas the *c-kit* gene is expressed in primordial germ cells⁶⁸; and (3) the *Sl* factor promotes the survival, but not the proliferation, of primordial germ cells in serum-free cultures^{63,69}. The *Sl* factor illustrates two important principles. First, whereas many signalling molecules that promote the survival of a cell also stimulate its proliferation, this is not always the case. Second, the *Sl* factor is produced in both soluble and plasma membrane-bound forms, and the membrane-bound form is much more effective both *in vivo*⁷⁰ and *in vitro*⁶⁹, indicating that cell-bound signals, as well as soluble ones, can promote cell survival.

Adult animals also need to be able to eliminate misplaced cells. When the skin is cut, for example, epidermal cells are displaced into the hypodermis and subcutaneous tissue, where they could cause trouble if they survived and proliferated; dependence on tissue-specific survival signals could, in principle, eliminate the problem by ensuring that the displaced cells die. The same mechanism may prevent cancer cells from establishing metastases; the protection would fail, however, once cancer cells, through mutation, became able to produce autonomously either their own survival factors, the intracellular signals stimulated by such factors, or other proteins, such as BCL-2 (refs 39–41), that can protect the cell from programmed cell death.

Competition for survival signals

Dependence on survival signals can also be useful in the control of cell numbers in higher animals, especially if cells are forced to compete with one another for limiting amounts of such signals. Developing sympathetic neurons provide an informative example. They are produced in larger numbers than are needed and then apparently compete with one another for limiting amounts of nerve growth factor released by the target cells they innervate⁷²; only some of the neurons get enough nerve growth factor to survive, while the others die^{11–13,49,50,73}. In this way the number of sympathetic neurons innervating a population of target cells is automatically adjusted to match the number of target cells. A similar competition for target-cell-derived survival signals (neurotrophic factors) seems to operate widely in the developing vertebrate peripheral and central nervous systems, where it is thought to play an important part in matching the numbers of presynaptic and postsynaptic cells during both evolution¹³ and development^{11–13,49,50}.

It seems unlikely that such a simple and effective mechanism for controlling cell number is confined to neurons. In addition to providing a mechanism for matching the numbers of different types of cells in a tissue or organ, competition for survival signals would be a simple way to ensure that cell turnover in adult tissues is balanced (as it must be if the tissue is neither growing nor shrinking) so that the number of cells produced by division is exactly matched by the number that die: if a given level of survival factor supports a certain number of cells of a particular type, any increase of these cells above this number would stiffen the competition and thereby tend to cause enough cells to die to return the cell number to its original value; a fall in cell number would have the reverse effect. This mechanism could explain the rapid regression of experimentally induced hyperplasia that has been observed to occur in various mammalian organs, such as the adrenal cortex, liver, kidney and pancreas; the regression occurs much too rapidly to be explained solely by a decrease in cell proliferation, and in several cases it has been shown to result from a large increase in apoptotic cell death^{7,74–77}. Although the regulation of cell numbers is usually considered mainly in terms of the control of cell proliferation,

these observations suggest that, even in mature animals, control of cell survival can also play an important part. It is possible that growth hormone and insulin-like growth factors stimulate vertebrate growth at least partly in this way: for some types of cells in culture, insulin-like growth factors promote cell survival rather than cell proliferation^{59,78–80}.

In addition to contributing to the control of cell numbers in a tissue or organ, competition for limiting amounts of survival factors could have the added advantage of continually selecting for the most competitive cells—a form of survival of the fittest. The most competitive cells might be those with most receptors for survival signals, those with the most efficient signal transduction pathways, and so on. The same type of selection process may also operate at the level of cell proliferation, with cells competing for limiting amounts of growth factors^{81,82}, but on its own this would not eliminate the less competitive cells as effectively as competition for survival factors. Intercellular competition comes at a price, however: it contributes to tumour progression⁸³ and is thus partly why one in five of us will die of cancer.

The way ahead

If many types of animal cells require signals from other cells to survive, then there is much work to be done: compared with the great amount of attention given to the control of cell proliferation, remarkably little attention has been given to the control of cell survival, other than in a small number of special cases, many of which I have mentioned. In addition to defining the molecular mechanisms of normal cell death, one will need to determine (1) the survival factors for each cell type; (2) the receptors for these factors; (3) the intracellular signalling pathways activated by the receptors; (4) how these pathways suppress cell death (is it always by suppressing cell suicide, for example?); and (5) how the amount of each survival factor is controlled. In short, much of what has been, and is being, done for cell proliferation control will need to be done for cell survival control. □

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ARTICLES

Palaeomagnetic constraints on the geometry of the geomagnetic field during reversals

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Palaeomagnetic records of the path of the pole during reversals of the Earth's magnetic field provide a test of the hypothesis that dipolar or low-order axisymmetric components of the field dominate during reversals. Multiple records of reversals during the past 12 Myr show no simple or consistent geographical pattern. Although a more robust analysis of the transitional field awaits a greater number of well-distributed sampling sites, the present data are not inconsistent with the simplest models, in which a field reminiscent of the non-dipole component of the present-day field becomes dominant.

SINCE the first palaeomagnetic records of geomagnetic reversals were published 30 years ago, there has been hope that detailed knowledge of field behaviour during transitions between the two polarity states would reveal key aspects of the geodynamo. The first results^{1,2} indicated that the directional changes occur over a short period (between 2,000 and 15,000 yr) and are systematically accompanied by a large drop (almost an order of magnitude) in the field intensity. A first attempt to compare the virtual geomagnetic pole (VGP) paths of the last reversal as seen from Japan and California³ led to the conclusion that the field was predominantly non-dipolar, a view which was later reinforced by additional data from the same transition.

Basically, three types of model were proposed to account for the non-dipolar structure of the reversing field. The first approach (referred to as the flooding model^{4–6}) involves time-dependent low-order zonal harmonics (that is, those symmetrical

about the Earth's rotation axis), produced by coexisting dipolar axial sources in the core. At some point of the reversal the transitional field will become vertical (upward or downward) at any site of observation; the VGP paths should thus pass near the site of observation or its antipode⁵.

The second approach (initially called 'the standing field model'³) suggests that a persistent component would dominate after collapse of the axial dipolar structure and is observed through successive reversals. Further developments of this model have been proposed recently by several authors^{7–9} who noticed a preferential grouping of VGP paths from different reversals over the Americas and to a lesser extent their antipode. After a first interpretation involving a dipolar transitional component, very recent work¹⁰ claims that a particular octupole component (h_3^1) could account for this characteristic. If so the VGP paths of multiple records relative to the same reversals from different sites should lie systematically within these two sectors of longitude.

Although many data obtained from sediments appear as longitudinally confined VGP paths, complex features such as large and rapid directional changes and rebounds of the directional vector to a previous direction are present in other records. Following early conclusions reached by Dagley and Lawley¹¹ from a review of volcanic data from Iceland, the diversity of these pole paths has led several authors to support a third kind of model in which the non-dipole field becomes dominant during the transition^{12,13}. This conclusion was reached independently from a statistical analysis of the present geomagnetic field¹⁴.

The large variety of models for geomagnetic reversals reflects the difficulty in using palaeomagnetic observations to constrain the geometry of the transitional field. Even more vexing is the fact that the fundamental question of dipole versus non-dipole dominance is not yet answered. It is often argued that there are currently too few transition studies to assess the validity of these

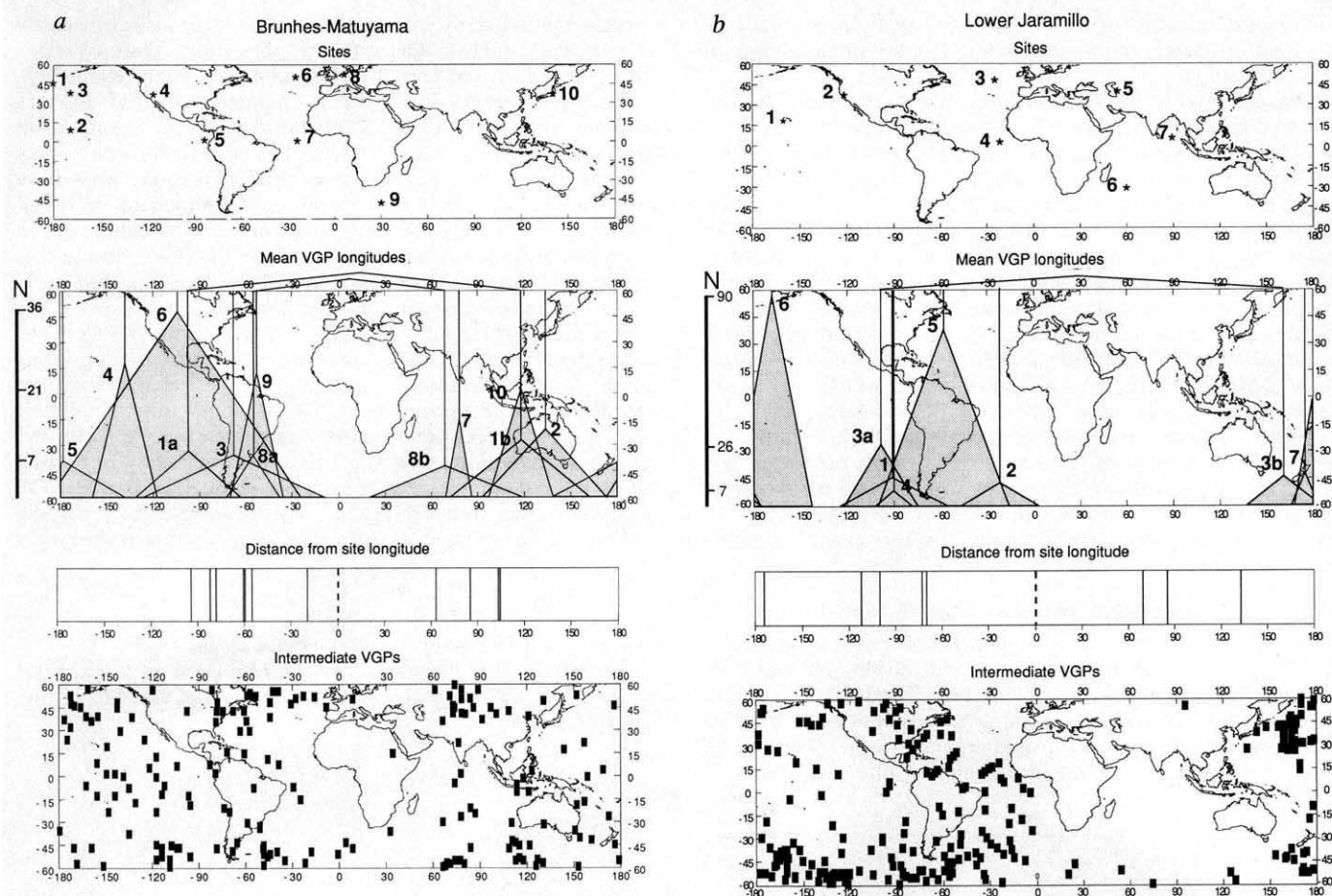
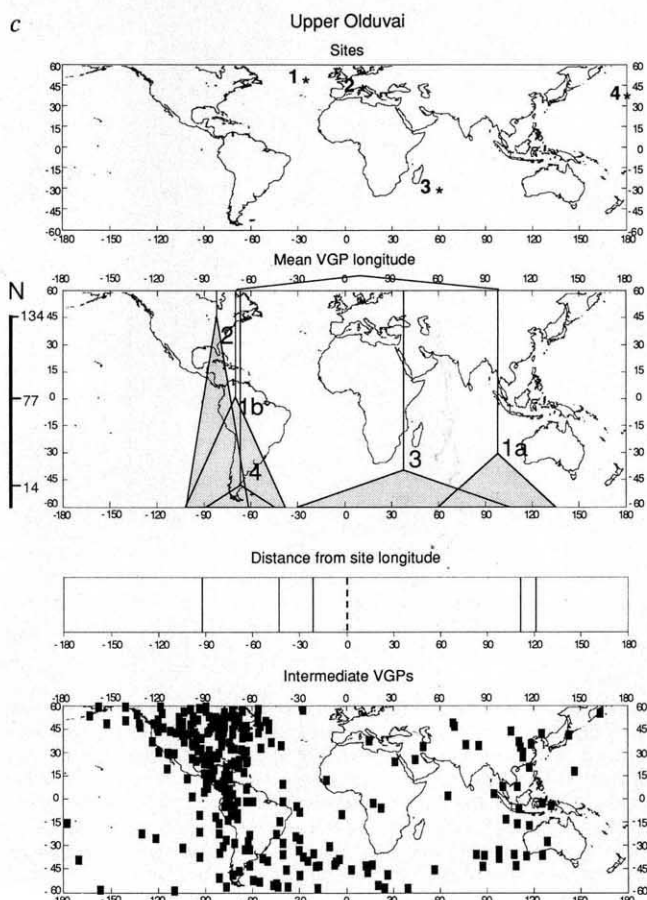


FIG. 1 Summary of transitional records for the three best-documented reversals younger than 2 Myr. *a*, Matuyama-Brunhes; *b*, lower Jaramillo; *c*, upper Olduvai. For each individual reversal separate maps give: the site distribution (top panel); the mean virtual geomagnetic pole longitudes (MVL); the distances of the MVLs from the site meridian (common site longitude), and the positions of the individual VGPs (bottom). Site numbers are given as a function of longitude. The height of the triangles attached to the MVLs is a function of the number of transitional VGPs indicated by *N* to the left of the map; the width of their base gives the dispersion around the mean (see text for definition).



models, but in fact many palaeomagnetic records have been published in the past few years. We will attempt to use available records to test several models. There exist multiple records of the five most recent reversals (Matuyama-Brunhes, upper and lower Jaramillo, upper and lower Olduvai) from various localities. The simple predictions of the different classes of models based on the longitude of the VGP paths allow us to discern whether the transitional field is consistent with an axial dipolar component, a zonal geometry or can be described in a systematic way reflected by preferential bands of longitude.

Selection of the records

Any attempt to compile published palaeomagnetic records requires that one set up criteria that define 'reliability'. In a first classification of reversals in terms of quality (A, B and C)¹⁵, the best records, classified as 'A', had at least one VGP at latitudes higher than 60° and more than one VGP below 60° in each hemisphere. These criteria were later extended so that a minimum of four intermediate directions was required, with stepwise demagnetization of all transitional samples and minimal analyses of the magnetic mineralogy¹⁶. Although such experimental control is clearly essential, investigations and interpretation of magnetic mineralogy are often not straightforward:

limiting the selection to studies involving stepwise demagnetization unfortunately results in an unreasonably small number of records.

One is left with the (testable) hope that at least some robust features of the transitional field are relatively insensitive to selection criteria used in compilations. This assumption is likely to be valid unless systematic trends are introduced by the recording processes of magnetization in the natural samples or by laboratory procedures used to isolate this magnetization. We have tested this approach by performing a two-step analysis, based mainly on the resolution of the records, which in turn depends on the number of intermediate directions and their distribution within the transition. We first selected all records of unambiguously identified complete polarity reversals younger than 12 Myr that satisfied the following criteria: they were published in refereed journals, had more than five intermediate VGP positions (with absolute value of latitude less than 60°) and had a reasonable number of full polarity directions preceding and following the transition. These records constitute our 'level 1'.

Those level-1 records that in addition have more than 15 intermediate VGPs represent our 'higher class' selection (level 2).

Records of reversals younger than 2 Myr

As we have said, particular emphasis has been given to the records obtained for the five most recent reversals, as summarized in Table 1a which lists the 25 'level-1' records. To obtain the same statistical weight and geophysical significance, different records of the same reversal from the same site were considered as a single datum. It is not surprising that this compilation is

largely dominated by sediments, as recent reversals can be easily identified through magnetostratigraphic study. Only two volcanic records are of level-1 quality and have been retained.

Each transitional VGP path is characterized by a different number of intermediate VGP positions. Any comparison between reversal records based on plots of individual poles may thus be strongly biased by the most detailed records. We believe that this happens in ref. 7. Because VGPs tend to be longitudinally confined, each VGP trajectory can be characterized by a mean longitude and the dispersion of VGPs around this mean meridian. These two parameters can be estimated simply as an arithmetic mean and its standard deviation, respectively. The mean VGP longitude (to be referred to as the MVL) of the best-fitting meridional great circle is more sensitive to positions closer to the Equator. Equatorial positions characterize the middle of the transition if the field is dominantly dipolar. It may therefore seem reasonable to weight each VGP_{*i*} by the cosine of its latitude ($\cos \lambda_i$). This was achieved by projecting the unit directional vectors (x_i, y_i, z_i) associated with every VGP position on the equatorial plane. The mean value of longitude (MVL) was calculated as the vector sum of these projections:

$$\text{MVL} = \tan^{-1} \left(\frac{\sum_{i=1}^n y_i}{\sum_{i=1}^n x_i} \right)$$

where n is the number of intermediate poles.

Thereafter, the dispersion may be estimated as the angular standard deviation, a simple two-dimensional statistic on the equatorial plane:

$$\delta = \cos^{-1} \left(R / \sum_{i=1}^n \cos \lambda_i \right)$$

where

$$R = \sqrt{\left(\sum_{i=1}^n x_i \right)^2 + \left(\sum_{i=1}^n y_i \right)^2}$$

But equatorial transitional directions tend to be those for which the intensity is a minimum, implying that the directions may not have been properly recorded and that the relative importance of possible undetected overprints is maximum. Therefore, we have recalculated mean longitudes and dispersions after replacing the $\cos \lambda_i$ weights first by 1, then by $\sin \lambda_i$. The calculations are robust enough (or the fit to a meridian is good enough) that discrepancies between these three calculations never exceed 5° and 10% for mean longitudes and dispersions, respectively.

We are well aware that these simple calculations do not provide a full statistical description of the data, which would require much additional and still unavailable information (related to the temporal control of the records, resolution in sampling and stratigraphy, variations in deposition rates, remaining overprints and so on); they simply yield a way of comparing the first-order configurations of the VGP paths and testing models dealing with longitudinally confined VGP paths, with a built-in normalization of data density.

The mean VGP longitudes obtained for the three best-documented of the five last reversals have been plotted in Fig. 1. VGPs are in some cases distributed within two separate longitudinal bands, and were then represented by two separate MVLs. This is of course a limitation and a breakdown of the hypothesis being tested. The degree of longitudinal confinement, as quantified by the parameter δ , is plotted as the base of triangles centred on every MVL (actually 2δ). The perpendicular height of the triangles gives the number of intermediate VGPs. This representation has the advantage of providing a quick and global view of data resolution, as well as the overall dispersion of directions within each record. A VGP path defined by a large number of intermediate positions restrained within a narrow band of longitude is depicted by a high and narrow triangle, whereas records with a few VGP positions scattered over a wide range of longitudes are reflected by short triangles with a wide base. Also shown are the locations of the sampling sites, the

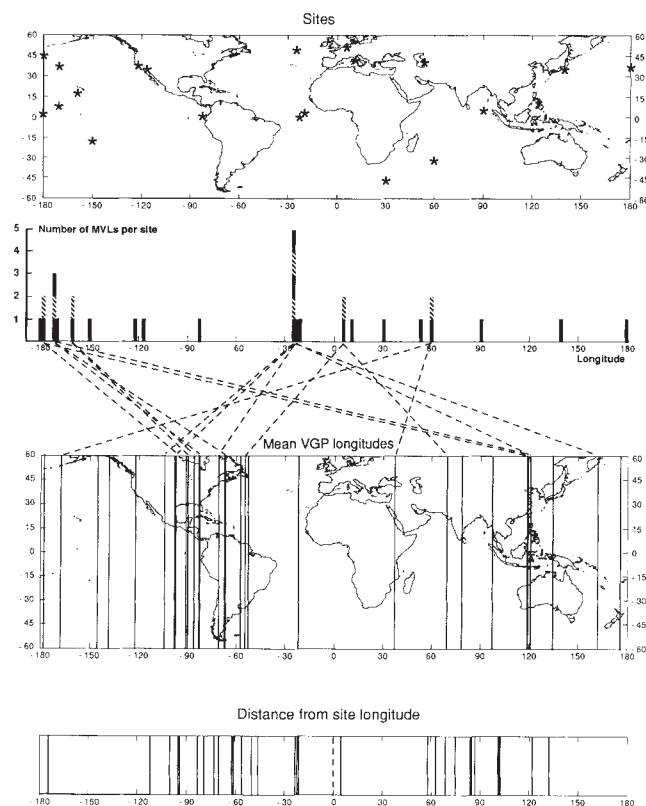


FIG. 2 Synthesis of the sampling sites and the MVLs for the five reversals younger than 2 Myr. In several cases records of different reversals were obtained at the same site; the number of VGPs has thus been plotted as a function of longitude. Some MVLs obtained from the same site (dashed lines) cluster over the Americas whereas others are found over distinct longitudes (not antipodal to the Americas). The majority of the MVLs are found more than 45° away from the site longitude. Similar results are obtained with level-2 data (but we should note that this 'higher class' selection does not incorporate the sites from the central Pacific).

distances of the individual MVLS from their site longitude and the individual VGP positions.

The Matuyama–Brunhes transition (Fig. 1a) is by far the best documented, with 10 independent studies. All but one site are located within two narrow latitude bands (0–10° N and 35–50° N) and there are no sites between 30° E and 140° E. The records are characterized by reasonable resolution (the number of VGPs ranges from 7 to 36) but their overall longitudinal scatter ranges from 8° to as high as 60°. Deep Sea Drilling Project hole 609B provides an outstandingly large number of VGPs, but these are very scattered within the entire hemisphere (only one VGP between latitudes –30° and 30° falls over the Americas). The MVLS, as well as the individual VGPs, seem to be rather uniformly distributed. Individual VGPs are scattered around the globe, except over Africa. When referred to a common site longitude^{4–6}, the MVLS are still rather uniformly scattered, although all but one (number 10) fall within 45° of the meridians located 90° away from the site on both sides.

Seven distinct records of the lower Jaramillo could be retained. The VGPs (Fig. 1b) are mostly confined to the western hemisphere, with a remarkable absence over Africa, Asia and the Indian Ocean. Four MVLS (1, 4, 3A and 5) are located over the American continent and three over the Pacific Ocean. Several individual VGP positions obtained from a volcanic record (number 2) are distributed along the western coast of Africa. One record (site 3) from the North Atlantic (again, hole 609B) is characterized by two ‘bands’ of longitudes, 100° away from each other. One MVL (3B) is located ~180° away from the site longitude; all others are within 45° of the meridians 90° away from the sites.

The four studies of the upper Olduvai show a preponderance of the individual VGP positions over the Americas (Fig. 1c)

which is largely due to a highly detailed single record (site 2, Po valley⁷) characterized by 135 intermediate VGPs. Only the Pacific Ocean seems to be devoid of intermediate poles. Two MVLS out of five fall within 45° of the site longitude or its antipode.

Only two records of the upper Jaramillo and two of the lower Olduvai meet our selection criteria. Although in each case the two records were obtained from nearby sites along the same meridian, they have strikingly different characteristics. The MVLS of the upper Jaramillo (including a detailed volcanic record from Tahiti) are significantly different from each other, one lying over the Americas and the other in the Pacific. Both trajectories of the lower Olduvai are mostly confined over the Americas, although three intermediate VGPs from core K78019 are located 140° away. The differences between the morphology of these last two records could be due to their rather poor resolution. Indeed, they do not satisfy the requirements for level 2. The longitude band from 70° W to 100° E is devoid of intermediate poles. The three MVLS are within 45° of the meridians 90° away from the sites.

A stack of all records from the five recent reversals is shown in Fig. 2. Twenty-five out of 30 MVLS (nearly 85%) fall within 45° of the two meridians located 90° away from the common site longitude. This observation leads us to reject the elegant suggestion by Hoffman⁴. In absolute geographical coordinates, there is a visual indication of more MVLS being concentrated on the Americas (say 50° W to 110° W) than elsewhere, as suggested by Laj *et al.*^{7,31}. Thirteen out of 30 MVLS (43%) lie in this band, and six (20%) in the antipodal band, for a total of 63% of the intermediate poles within these two 60° wide bands of longitude. We need to test, however, whether the observed distribution of the 30 MVLS around the globe is indeed

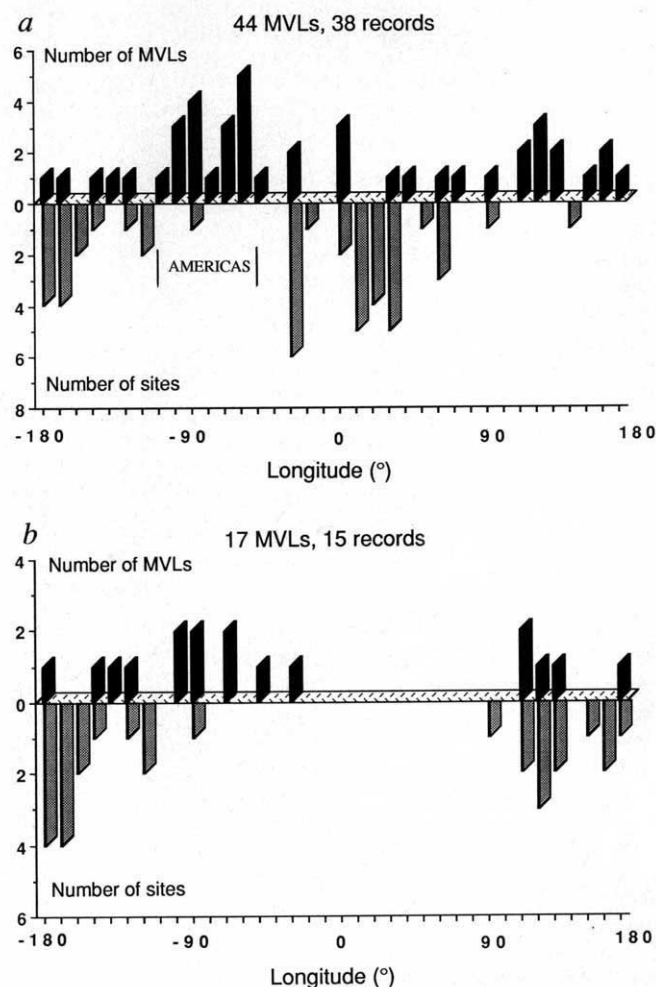


FIG. 3 Histogram of the MVLS (upper part of each plot) and site longitudes (lower part) within 10° longitude bands relative to the reversals younger than 12 Myr listed in Table 1a and b. a, All level-1 data (more than five intermediate VGPs) b, level-1 data but after removal of eastern Atlantic and western European sites. All plots, including level 2 data not shown here, point to the same features in the MVL distribution.

TABLE 1 Characteristics of the reversal records

(a) Reversals younger than 2 Myr											
Reversal	Age (Myr)	Locality	MVL number	Site latitude	Site longitude	N VGPs	MVL	MVL- site	δ	Ref.	Remarks
Brunhes-Matuyama	0.73	V20107 + V20108	1A	45	-180	9	-97	83	35	17	2 bands of longitude
	0.73	V20107 + V20108	1B	45	-180	11	118	-62	44	17	
	0.73	K78 1019	2	9	-170	13	134	-56	41	18	
	0.73	North Pacific	3	38	-170	8	-68	102	61	19	
	0.73	Tecopa	4	35	-117	26	-138	-21	23	16	
	0.73	RC1521	5	1	-83	7	-178	-95	43	17	
	0.73	DSDP609B	6	48	-25	36	-104	-79	84	20	
	0.73	ODP 604D	7	0	-23	15	78	101	8	13	
	0.73	Bruggen	8	51	6	8	-55	-61	17	21	2 bands of longitude
	0.73	Bruggen	8B	51	6	6	69	63	49	21	
	0.73	V16-58	9	-47	30	24	-53	-83	17	9	
	0.73	Boso Peninsula	10	35	140	21	118	-22	23	22	
Upper Jaramillo	0.91	K78 030	1	18	-160	15	-86	75	45	23	
	0.91	Tahiti	2	-17	-150	24	-145	5	52	24	volcanics
Lower Jaramillo	0.98	K78 030	1	18	-160	12	-91	69	34	23	
	0.98	Clear Lake	2	38	-122	10	-22	-100	25	25	volcanics
	0.98	DSDP 609B	3A	48	-25	26	-98	-73	24	20	2 bands of longitude
	0.98	DSDP 609B	3B	48	-25	12	161	-174	24	20	
	0.98	ODP 665A	4	3	-20	7	-90	-70	12	26	
	0.98	Adzidhere	5	40	54	75	-58	-112	43	27	
	0.98	RC1414	6	-32	60	90	-168	132	26	28	
	0.98	ODP 758B	7	5	90	14	175	85	9	26	
Upper Olduvai	1.66	DSDP 609B	1A	48	-25	36	97	122	38	20	2 bands of longitude
	1.66	DSDP 609B	1B	48	-25	77	-71	-46	32	20	
	1.66	Po Valley	2	42	11	134	-83	-94	20	7	
	1.66	RC1414	3	-32	60	26	37	-23	68	29	
	1.66	K7501	4	37	180	14	-68	112	22	23	
Lower Olduvai	1.87	K78 113	1	2	-180	8	-122	58	23	23	
	1.87	K78 1019	2A	9	-170	12	-83	87	11	30	2 bands of longitude
	1.87	K78 1019	2B	9	-170	3	120	-50	14	30	

(b) Reversals from 2 to 12 myr											
Reversal	Age (Myr)	Locality		Site latitude	Site longitude	N VGPs	MVL	MVL- site		Ref.	Remarks
Gauss-Matuyama	2.45	Turkmenia		40	60	100	130	70		32, 33	
Gauss-Matuyama	2.45	Searles Valley		35	-120	16	-50	70		34	
Lower Nunivak	3.97	Italy		38	10	17	0	-10		35	
Upper Sidujfall	4.10	Italy		38	10	40	0	-10		35	
Lower Sidujfall	4.26	Italy		38	10	5	40	30		35	
Upper Thvera	4.39	Italy		38	10	25	-70	-80		35	
KP102-KP402	5.67	Crete		35	20	26	160	140		36	
KP101-KP401-KS06	5.87	Crete		35	20	120	-60	-80		36	
KS05	6.34	Crete		35	20	25	120	100		36	
KS02	7.25	Crete		35	20	24	-60	-80		36	
ZM03	11.16	Zackinthos		34	30	24	150	120		37	
ZM02	11.64	Zackinthos		34	30	52	120	90		37	
ZM01	11.76	Zackinthos		34	30	70	-30	-60		37	2 bands of longitude
ZM01	11.76	Zackinthos		34	30	12	0	-30		37	

(c) Volcanic records											
Reversal	Age (Myr)	Locality		Site latitude	Site longitude	N VGPs	Ref.	Site number (Fig. 4)			
Brunhes-Matuyama	0.73	Tahiti		-17	210	5	24	2			
Upper Jaramillo	0.91	Tahiti		-17	210	24	24	2			
Lower Jaramillo	0.98	Clear Lake		38	238	10	25	4			
N-R	2.9-3.1	Huahine		-16	210	15	38	2			
R-N	3.8-5.1	Hawaii		22	200	7	39, 40	1			
N-R	3.8-5.1	Hawaii		22	200	15	39, 40	1			
4R-N + 2N-R	Tertiary	E. Iceland		65	345	38	11	5			
R-N	Tertiary	W. Iceland		64	340	17	41, 42	5			
R-N	15.3	Oregon		42	241	45	43	3			

Parts *a* and *b* show sedimentary and volcanic records; *c* shows volcanic records only. *N* VGPs is number of intermediate VGPs (between 60° N and 60° S); MVL, mean VGP longitude; MVL-site, distance between the MVL and the site longitude; δ , angular standard deviation.

statistically different from a uniform (random) distribution. We first divided the sphere into 18 sectors of 20° width in longitude and found the χ^2 statistic of the departure from a uniform distribution to be 19.2. The critical value for the test at the 5% significance level with 16 ($n-2$) degrees of freedom is 26.3. Therefore, there seems to be no reason to reject the hypothesis of a uniform MVL distribution. But the numbers of MVLs in some classes (and the total number of MVLs) are small enough that one may worry about the statistical significance of the test. If the number of sectors is reduced to nine (each 40° wide) the test is barely significant at the 5% level ($\chi^2=14.4$, whereas critical $\chi^2_c=14.1$). With only six sectors of longitude, the test becomes very sensitive to the position of the sector boundaries and is thus not robust. If for instance a 60° sector of longitude is chosen to coincide exactly with the 60° sector over the Americas, where there is the largest visual concentration of MVLs, the null hypothesis can be rejected ($\chi^2=21$; $\chi^2_c=11.1$) whereas a 30° offset of the sectors allows one to retain the null hypothesis ($\chi^2=10$). There is of course a trade-off, and the hypothesis of preferential bands of longitude becomes less meaningful (and less attractive) as the width of bands is increased.

Records of reversals younger than 12 Myr

Forty-four MVLs of distinct reversals younger than 12 Myr (beyond this limit the data are too sparse) are included in the

level-1 selection (requiring at least five intermediate VGPs), while 24 remain in the level-2 selection (at least 15 VGPs) (Table 1a and b). In Fig. 3, we show the distribution of MVLs within 10° longitude bands, together with the distribution of sampling sites. The MVLs are not uniformly distributed around the sphere and can be roughly classified as three density maxima in the longitude bands around 0°, 120–180° E and 40–110° W.

Perhaps the most striking feature apparent in Fig. 3a is the very poor distribution of sampling sites, largely dominated by eastern Atlantic and western Europe (30° W to 70° E) and to a lesser extent by mid-Pacific records (120 to 180° W). There is a remarkable absence of sites within the longitudes of America (50 to 100° W) and Asia (80 to 180° E). Therefore, one wonders whether the distribution of the MVLs could be biased by the European and Atlantic records. The first-order influence of these sites can be estimated by removing these records from the selection, markedly enhancing the importance of the Pacific sites (Fig. 3b). The distribution of the remaining MVLs is not much changed, although the number of points within each longitude class is too small (no more than two) to draw statistically robust conclusions. The most notable observation is the total disappearance of MVLs between longitudes 0 and 90° E, which reveals that only European and Atlantic records have MVLs within this sector. On the other hand, there are still two bands of MVLs (not really antipodal) over the Americas and eastern Asia. Another approach to explore the influence of site

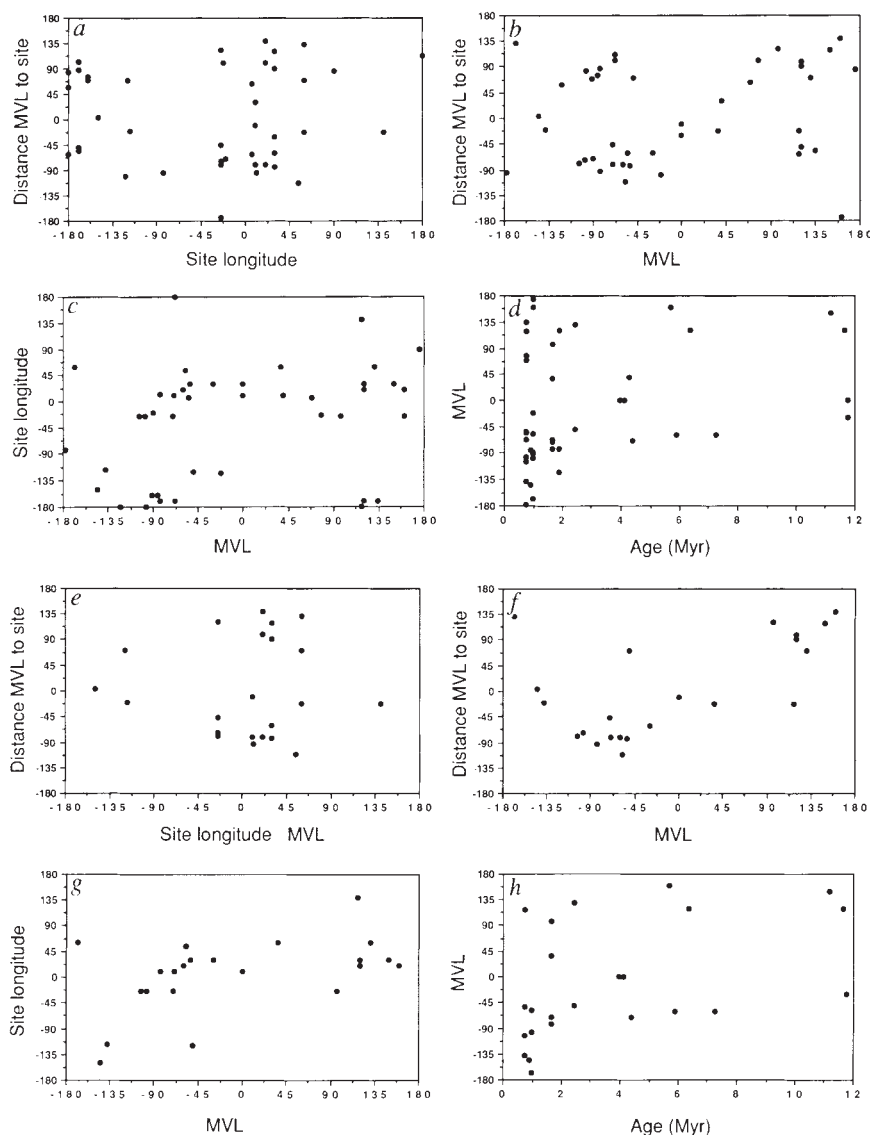


FIG. 4 Characteristics of transition paths of level 1 (a to d) and level 2 (e to h) quality for reversals younger than 12 Myr in terms of MVLs, distances of MVLs from site longitudes and site locations. The same features, especially the lack of any significant correlation, are revealed by both series of plots. Interestingly, most of the MVLs found over the American continent lie more or less 90° away from the site meridian (b, f and c, g). There is no age-related pattern, however, in the configuration of the MVLs (d, h).

coverage was to sample randomly one site in each 10° band of longitude. Unfortunately, many longitudinal bands are then empty. Successive random samplings yielded similar distributions, characterized by a rather uniform repartition of the MVLs within the previous longitudinal bands.

Another illustration of the relationship between the site locations and the MVLs is given by the plots in Fig. 4. Regardless of the data selection used, the distances of the VGP paths from their respective sites are not correlated with the site locations (Fig. 4a and e) and any configuration seems to be possible at any longitude. There are, however, some interesting features in the plot of the distance (difference) between the site and mean VGP longitudes as a function of MVL (Fig. 4b and f). The MVLs found over the American continent (30 to 115° W) lie ~90° away from their site meridians and thus belong either to the eastern Atlantic/western Europe sector or to the mid-Pacific (Fig. 4c and g). Other MVLs recorded from these two areas are widely distributed around the globe. Therefore, should the VGP paths found over the Americas result from a low-order harmonic component, they would at least be confined to certain periods

of time. Figure 4d and h shows that such has not been the case.

It is interesting that all the characteristics described above remain globally unchanged with any of the data selections (in addition to our levels 1 and 2, we calculated the same statistics for a number of other selection criteria such as the number of VGPs per 10° band of latitude and longitude). We also resumed the χ^2 tests summarized in the previous section, this time with 44 rather than 30 MVLs. In all cases the conclusion in favour of the null hypothesis was made stronger, and in the only case when the null hypothesis could previously be rejected (the 60° sectors centred on the Americas), the test became less significant. Therefore, the presence of additional data seems to reinforce the case for a uniform MVL distribution. The apparently random distribution of the MVLs implies a complex structure for the transitional field. On the other hand, a complete assessment of the data set should consider the fact that recording processes in sediments may introduce certain artefacts (smoothing of the signal due to post-depositional reorientations, inclination error, chemical processes and so on). Under these conditions, it is bound to be difficult to distinguish between random noise inher-

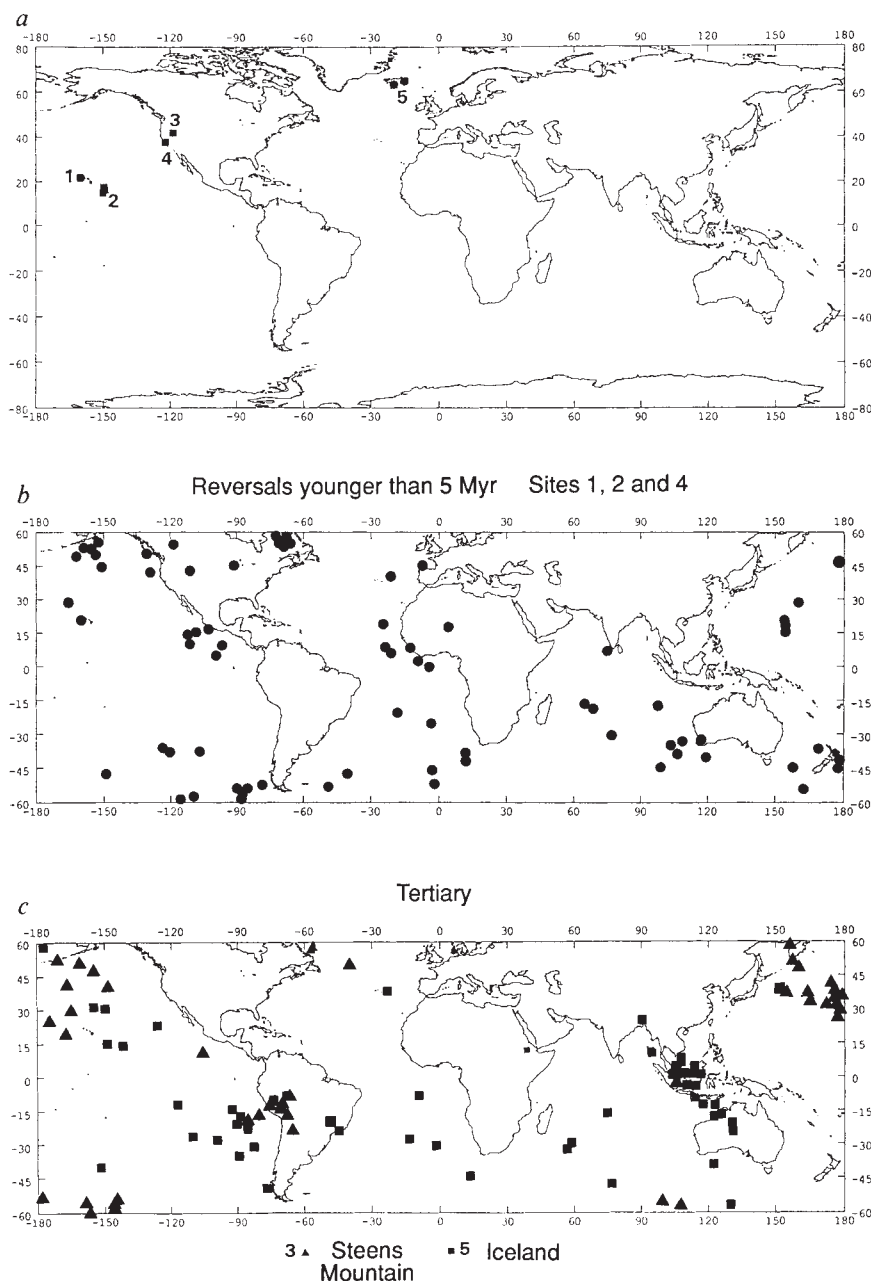


FIG. 5 Palaeomagnetic records of 10 reversals from lava flows from five volcanic sequences. *a*, Location of the sites (see refs in Table 1c). Only three distinct areas (central Pacific, western North America and Iceland) are actually covered by data; *b*, individual VGP positions obtained from three younger sequences located in the mid-Pacific and the northwest of the United States; *c*, individual VGP positions for older records (Steens Mountain and Iceland). Although the records were obtained from relatively close areas the absence of any coherent pattern emerging from the transitional VGPs does not suggest persistent or reproducible features of the transitional field.

ent in the recording process and typical variations of the non-dipole field. A pessimistic assumption would be that the apparently random distribution of the MVs over the globe reflects a high noise level in the records. A partial test of this can be obtained by comparing the characteristics of sedimentary and volcanic records.

Volcanic records

Many volcanic records are not clearly identified and/or are incomplete (without VGP paths) so that only two studies could be included in our analysis. Relaxing our selection criteria, we think that it is worthwhile to consider the distribution of the individual VGPs from 10 studies summarized in Table 1c. The geographical distribution of the sites is restricted to three areas (mid-Pacific, western America and Iceland). The data shown in Fig. 5 have been arbitrarily separated into two plots for younger and older reversals. Both plots show wide longitudinal scatter with some concentrations of VGPs within certain areas (for example around Indonesia), which could correspond to intense volcanic activity during short periods of time.

The studies of the Steens Mountain from Oregon (site 3)⁴³ and the upper Jaramillo from Polynesia (site 2)²⁴ are the two best-documented and complete volcanic records published so far. It is striking that both trajectories display large longitudinal loops similar to variations of much smaller amplitude reported in one of the most detailed sedimentary records (KS06, Crete⁴⁴). Such features are compatible with drifting sources of the non-dipole field and can be successfully reproduced with a recent model^{45,46} involving low-order zonal and drifting non-dipole harmonics. But the available palaeomagnetic records reviewed here do not support the presence of non-dipole low-order zonal harmonics; the transitional field may be at least as complicated as the present non-dipole field. The amplitudes of the loops observed in the volcanic records range between 100° and 180°, whereas they are considerably smaller in sediments. Volcanic samples provide us with a direct estimate of the variations of the non-dipole field during transitional periods. The averaging time inherent in the remanence acquisition in sediments implies a low-pass filtering effect which limits the resolution of details of the non-dipole field. Comparison of both data sets should yield an estimate of the degree of smoothing. The fact that similar features (even with dissimilar amplitudes) emerge from both kinds of records emphasizes their joint importance for our understanding of the transitional field.

Conclusion

Our principal conclusions can be summarized as follows: (1)

different records of a single transition do not show a unique common VGP path and are thus not compatible with a dipolar transitional field; (2) there is no statistically robust evidence for a preferred sector of longitude in the distribution of mean VGP paths; (3) there is no systematic relationship between the configuration of the VGPs and the site longitude (nor with the antipodal meridian), as predicted by models involving zonal non-dipole terms⁴⁻⁶; (4) rather, the VGP paths of sites confined to the longitudinal band of the Americas tend to lie 90° away from their site longitudes, which are located either in Europe or in the Pacific (the records from this last area are less detailed and could not be included in our 'higher class' (level-2 selection)); (5) the absence of any age-related pattern in the distribution of the VGP paths rules out the hypothesis of a persistent component of the transitional field.

Should preferential grouping over the Americas and confinement of records to ~90° away from site longitude be considered real, we wish to emphasize that the two are not compatible. In any case, about twice to three times as many MVs as are available today would be needed to allow more robust conclusions from the statistical tests. We conclude that the present data sets do not contain strong indications for a high degree of organization in the configuration of the geomagnetic field during reversal. The site-to-site variability of the MVs, as well as the large scatter of the VGPs from volcanic records, suggests that the transitional field is dominated by non-dipole components. The presence of large loops in the most detailed volcanic and sedimentary records shows some similarity to secular variation of the present non-dipole field, bringing us back to some early suggestions¹¹. Indeed, drifting and diffusing non-dipolar features would generate a random (and uniform, if reversal time is somewhat longer than drift rate) pattern of MVs. If this interpretation is correct, a very large number of detailed records of the same reversal from multiple sites will be required for a reliable description of transitional field geometry. *Note added in proof:* The values of the χ^2 test given here have been calculated without using the Yates correction. One referee pointed out that the values of the χ^2 test would be more significant if the sectors of analysis had been 'tuned' to the preferred longitudinal band of 45° centred on the Americas. We agree that in this case the result of the test ($\chi^2 = 15.46$) is slightly above the critical value of 14.1; but an offset of only 5° of the sectors allows one to retain the null hypothesis ($\chi^2 = 10.5$). The value of χ^2 falls to 12.9 if a single point is removed from the band of longitude of the Americas. Clearly the results are very sensitive to the number of data points and placement of sectors boundaries. □

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DNA recognition by GAL4: structure of a protein–DNA complex

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A specific DNA complex of the 65-residue, N-terminal fragment of the yeast transcriptional activator, GAL4, has been analysed at 2.7 Å resolution by X-ray crystallography. The protein binds as a dimer to a symmetrical 17-base-pair sequence. A small, Zn²⁺-containing domain recognizes a conserved CCG triplet at each end of the site through direct contacts with the major groove. A short coiled-coil dimerization element imposes 2-fold symmetry. A segment of extended polypeptide chain links the metal-binding module to the dimerization element and specifies the length of the site. The relatively open structure of the complex would allow another protein to bind coordinately with GAL4.

THE yeast protein GAL4 activates transcription of genes required for catabolism of galactose and melibiose^{1–3}. The DNA sequences recognized by GAL4 are 17 base pairs (bp) in length^{4–6}, and each site binds a dimer of the protein⁷. Four such sites, similar but not identical in sequence, are found in the upstream activating sequence (UAS_G) that mediates GAL4 activation of the GAL1 and GAL10 genes, for example⁸.

Functions have been assigned to various parts of the 881-amino-acid GAL4 protein (Fig. 1a), including DNA binding (residues 1–65)^{9,10} and dimerization (residues 65–94)⁷. The structure we describe here reveals a weak dimerization function in residues 50–64. In addition, three acidic activating regions have been identified (residues 94–106; 148–196; 768–881)^{11,12}, as has a region near the carboxyl terminus that binds the inhibitory protein GAL80 (refs 13, 14).

The DNA-binding region of GAL4 has six cysteine residues, conserved among a set of homologous proteins, that coordinate two Zn²⁺ ions in a bimetal–thiolate cluster^{15,16}. Two-dimensional NMR spectroscopy has been used to determine the structure of the free amino-terminal region in solution. As described in two accompanying papers^{17,18}, residues 10–40, which form the metal-binding domain, are a compact globular unit. Residues 1–9 and residues C-terminal to 41 are disordered.

We now report a crystallographic analysis at 2.7 Å resolution of a 65-residue fragment, GAL4 (1–65), bound specifically to DNA. The 19-bp DNA fragment contains the 17-bp palindrome that is a consensus of the 11 known GAL4 binding sites^{4–6}. The structure reveals a dimer of GAL4 (1–65) bound to each 17-bp site. The small zinc-binding module at the N terminus of each subunit lies in the DNA major groove, in contact with three base pairs at the extreme ends of the site. An extended linker segment of nine residues connects the zinc domain to a short coiled-coil, which forms a dimer contact in the complex. GAL4

(1–65) is a monomer in the absence of DNA. The open features of the complex, in which a long stretch of DNA at the centre of the 17-bp site is accessible in the major groove, suggest that another protein may be able to bind coordinately with GAL4.

Structure determination

Crystals in space group *P*₄₃₂₁₂ were prepared as described in the legend to Table 1. The structure of a Cd²⁺-containing complex was determined and refined, because the crystals were of better quality than the isomorphous crystals containing Zn²⁺. Isomorphous derivatives were obtained either by replacing Cd²⁺ with Zn²⁺ or Hg²⁺, or by preparing duplex DNA in which 5-iodo-uridine was substituted for thymidine in selected positions (Fig. 1; Table 1).

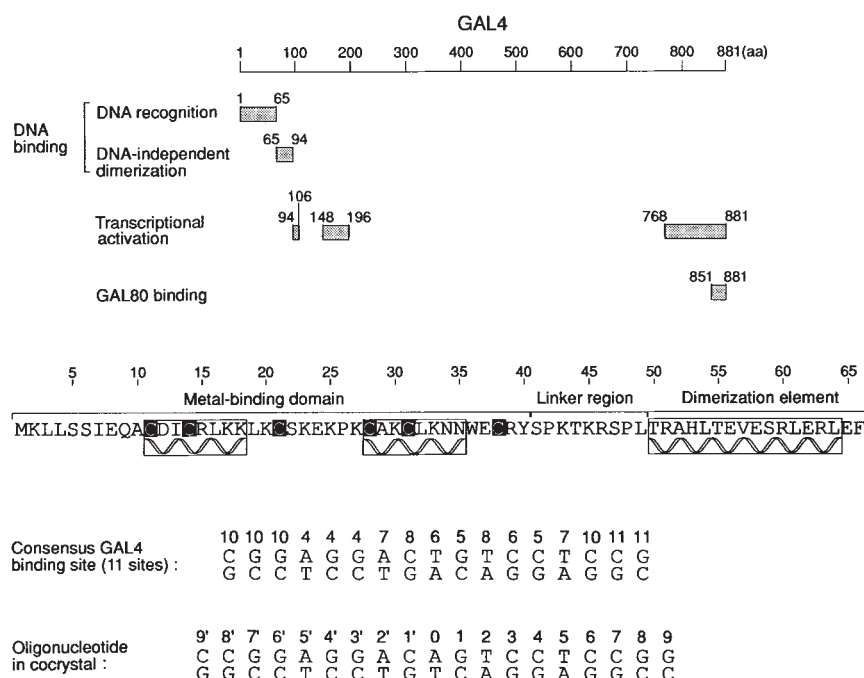
The structure of the cadmium-containing complex was initially determined to 3.2 Å by multiple isomorphous replacement (MIR) using phase information from one Hg²⁺ and four 5-iodo-uridine derivatives (Table 1). Locations of the heavy atom derivations confirmed that there was one complete protein–DNA complex per asymmetric unit, and that the protein bound the consensus DNA site as a homodimer. The initial MIR map showed clear density for B-form DNA, and the highest peaks in the map confirmed earlier spectroscopic experiments indicating that each protein monomer bound two closely spaced metal ions¹⁶. But the protein chain could not be traced. The map was improved by non-crystallographic averaging about a dyad relating the two protein–DNA half-sites¹⁹. The initial dyad was calculated using heavy-atom positions. Base pairs with ideal B-DNA geometry were built into the twofold averaged map using the model-building program FRODO²⁰. The DNA model was then refined, using the program CORELS²¹, with 'observed' structure factors calculated from averaged MIR density enveloped to contain only the DNA. Phases calculated from the refined DNA structure were combined with MIR phases, and the map derived by combining these phases with observed amplitudes was averaged, using an improved dyad calculated from heavy-atom and refined DNA phosphate positions. The averaged map showed a clear boundary between macromolecule and solvent, allowing construction of a molecular envelope for several cycles of phase averaging and solvent flattening. This improved the map, allowing an unambiguous trace for about 70% of the protein chain (Fig. 2).

Conventional positional refinement was then carried out to 3.2 Å resolution, using CORELS²¹ and XPLOR^{22,23}. Data to 2.7 Å were obtained from an iodine derivative of the complex at –155 °C (Table 1) and used for all subsequent refinements. The cell dimensions for the new crystal were ~1% smaller in each direction. The partially refined structure at –10 °C was fitted to the new data by rigid-body refinement in stages starting at 10–6 Å resolution and ending at 10–3 Å. Conventional positional refinement and simulated annealing were then carried out with XPLOR^{22,23}, after application of an anisotropic B-factor correction. Phases calculated from the refined model were improved by non-crystallographic symmetry averaging, and the resulting map was of sufficient quality to build most of the rest of the protein. Each part of the model was checked for errors by calculating phases from structures with omitted segments (between 4 and 10 residues on both sides of the

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FIG. 1 GAL4 and its DNA binding site. *a*, Functional

organization of intact GAL4^{7,9-13}. *aa*, Amino acids. *b*, Amino-acid sequence of the DNA-binding fragment of GAL4 used in the crystallographic analysis. Modular structure of the protein fragment is indicated above the sequence. Cysteine residues involved in chelating metal are shown as reverse white on black type, and helical regions are indicated by curly lines below sequence. This fragment, which contains the N-terminal 65 amino acids of GAL4 plus a C-terminal Phe derived from the cloning construct, was overexpressed in *Escherichia coli* using a Tac promoter. Cells were grown at 37 °C to an absorbance of 0.7 at 595 nm induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 2.5 h, and collected. Cadmium (acetate)₂ or zinc (acetate)₂ at a concentration of 100 μM was also added during the induction, and all subsequent purification steps were carried out in the presence of 10 μM of the respective metal. Following sonication, nucleic acid was removed by 2% streptomycin sulphate precipitation, and the protein was partially fractionated by ammonium sulphate precipitations at 35 and 65%. The GAL4 derivative, found predominantly in the 65% ammonium sulphate fraction, was dissolved and dialysed against low-salt buffer (20 mM HEPES, pH 7.5, 0.15 M NaCl, 10 μM cadmium (acetate)₂ or zinc (acetate)₂, 1 mM 2-mercaptoethanol, 100 μg ml⁻¹ PMSF, 10% glycerol) and fractionated on a Heparin-Sepharose column with a linear 0.15–0.6 M NaCl gradient. The peak eluting at ~0.3 M NaCl was further fractionated on a Cibacron blue column using the same salt gradient. Peak fractions were concentrated to ~40 mg ml⁻¹ for crystal-



lization. *c*, Upper panel, consensus GAL4 binding site with the degree of conservation indicated above the most conserved base. Lower panel, DNA fragment (19 bp) used in the crystallographic analysis, with the numbering scheme used in the text indicated above the base.

TABLE 1 Summary of crystallographic analysis

	Native 1	Native 2						
Metal ion	Cd ²⁺	Cd ²⁺	Zn ²⁺	Hg ²⁺	Cd ²⁺	Cd ²⁺	Cd ²⁺	Cd ²⁺
DNA fragment*	WT	IU55	WT	WT	IU0	IU2'	IU22'	IU55'
Resolution (Å)	3.2	2.7	3.2	3.2	3.2	3.8	3.2	3.2
Redundancy	7.3	4.2	4.0	1.3	2.9	3.2	4.1	3.9
Data coverage (%)	98	94	90	67	86	93	93	98
R _{sym} (%)†	9.8	7.5	10.7	9.5	12.3	9.1	11.0	9.5
MIR analysis								
Resolution (Å)	10–3.2	10–3.2	10–3.2	10–3.8	10–3.2	10–3.2		
Mean isomorphous difference‡			14.2	18.1	14.2	14.1	16.2	16.4
Phasing power§			—	—	1.3	1.7	1.7	1.8
Mean figure of merit					0.59 (overall)			
Refinement								
Resolution (Å)		8–2.7						
R factor (%)		23.0						
Reflections (F > 16)		6,411						
Total number of atoms		2,200						
Water molecules		51						
r.m.s. bond length (Å)		0.015						
r.m.s. bond angle (degrees)		2.9						
r.m.s. thermal parameter (Å ²)		2.2						

GAL4 (1–65) was expressed and purified as described in the legend to Fig. 1. Oligonucleotides and related heavy-atom derivatives were synthesized on a Milligen DNA synthesizer and purified by reverse phase HPLC using a Dynamax 300 column (Rainin). Crystals of GAL4 (1–65) bound to a blunt-ended 19-bp oligonucleotide containing the consensus 17-bp site from the UAS₆ (Fig. 1) were obtained by vapour diffusion, using 5-μl hanging drops containing 0.6 mM protein dimer, 0.9 mM DNA duplex, 16% MPD (2-methyl-2,4-pentanediol), 75 mM CaCl₂, 24 mM NaCl, and 12 mM sodium cacodylate, pH 6.8, equilibrated with a reservoir containing 27% MPD, 125 mM CaCl₂, 40 mM NaCl, and 20 mM sodium cacodylate, pH 6.8. Crystals grew in several weeks to a typical size of 0.4 × 0.2 × 0.2 mm. The crystals form in the space group P4₃2₁2 with *a* = *b* = 81.75 Å, *c* = 74.61; the cell shrinks to *a* = *b* = 80.85, *c* = 73.70 when frozen at liquid nitrogen temperature for high-resolution data collection. All data were collected at –10 °C except those designated 'Native 2', which were collected at –155 °C. All diffraction data were collected on a Siemens area detector.

* IU indicates 5-iodo-uridine has replaced thymine at the positions indicated (see Fig. 1c); WT, wild type.

† $R_{sym} = \sum_h \sum_i |I_{hi} - \bar{I}_h| / \sum_h \sum_i I_{hi}$, where I_{hi} is the mean intensity of the *i* observations of reflection *h*.

‡ Mean isomorphous difference = $\sum |F_{PH} - F_P| / \sum F_{PH}$, where F_{PH} and F_P are the derivative and native structure factor amplitudes, respectively.

§ Phasing power = $[(F_{H(calc)})^2 / (F_{H(obs)} - F_{H(calc)})^2]^{1/2}$.

|| The r.m.s. bond length and r.m.s. bond angle are the respective root-mean-square deviations from ideal values.

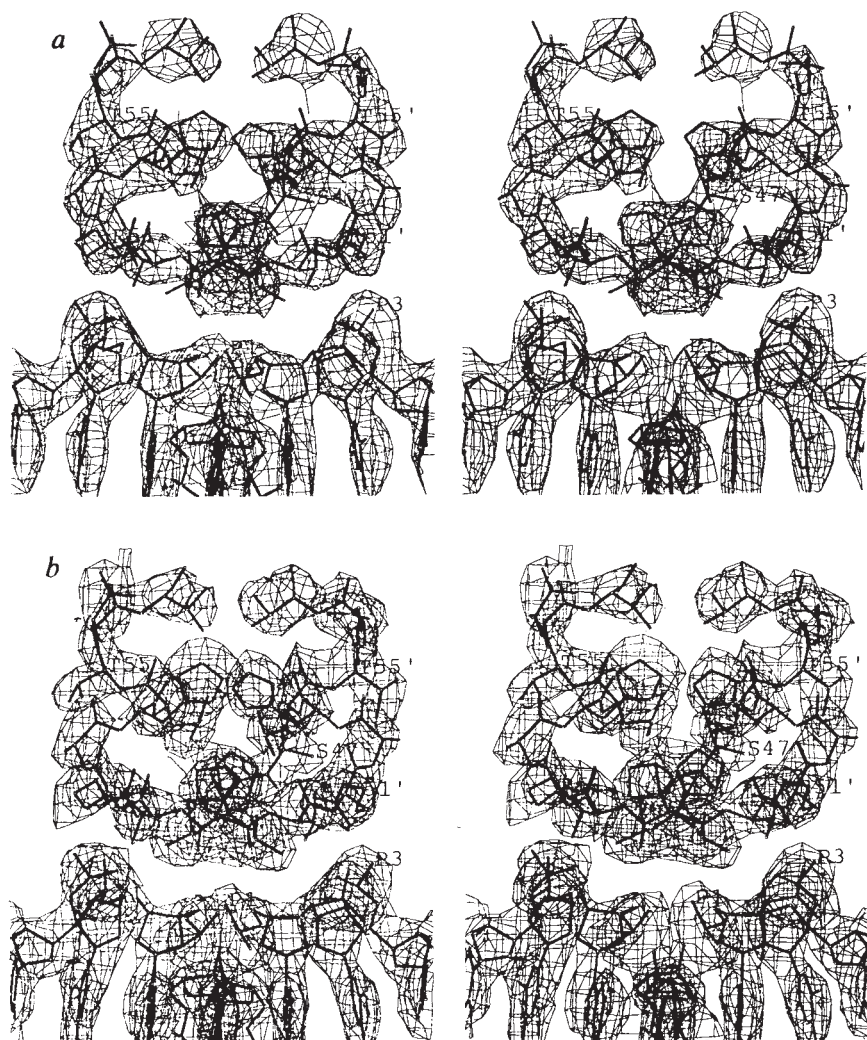


FIG. 2 Electron density map of the complex. *a*, Stereoscopic view of the part of the dimerization element of the protein that abuts the minor groove of DNA. Residues from the 2-fold-related subunit are primed. The map was calculated to 3.1 Å resolution after combining MIR and DNA model phases followed by four cycles of non-crystallographic phase averaging. The map is contoured at 1σ . *b*, $2Fo - Fc$ map (2.7 Å) of the refined model. The contour level is 1σ .

non-crystallographic dyad), refining these phases by twofold averaging, and using the phase-averaged maps to rebuild omitted regions. Refinement was extended to 2.7 Å using simulated annealing with XPLOR^{22,23}, followed by tightly restrained B-factor refinement. The model was rebuilt with reference to both averaged and unaveraged maps, and solvent molecules were added. Several more cycles of simulated annealing and B-factor refinement, followed by rebuilding and a last round of refinement with the program TNT²⁴, resulted in a final structure with reasonable geometry and a crystallographic *R* factor of 23.0%, using all reflections between 8 and 2.7 Å (Table 1).

Structure description

Overall view of the complex. The protein fragment binds to its DNA site as a symmetrical dimer. Each subunit folds into three distinct modules: a compact, metal-binding domain (residues 8–40), an extended linker (41–49), and an α -helical dimerization element (50–64). Residues 1–7 and 65–66 are disordered. An overall view of the complex (Fig. 3) shows that a large part of the DNA major groove is not contacted by the protein. The DNA is relatively straight. A metal domain lies in the major groove near each end of the DNA fragment. The paired parallel helices of the dimerization element project away from the DNA along the 2-fold axis of the complex. The metal-binding domain contacts three DNA base pairs in the major groove, and we therefore refer to it as a 'recognition module.'

Metal-binding domain. The recognition module is held together by two metal ions, tetrahedrally coordinated by the six cysteines

(Fig. 4). Two of the cysteines (11 and 28) ligate both metals, creating a 'binuclear cluster'^{17,25}, similar to the one first seen in metallothioneine^{26,27}. The folded polypeptide chain contains two short α -helices, each followed by an extended strand. Each helix connects to its strand through a structure often present at the C-termini of α -helices. The last helical residue has a positive ϕ angle, usually found only in glycine, but in neither helix is the last residue a glycine. The first and fourth residues of each helix are cysteines, as is the third residue of each succeeding strand. As this repetitive pattern suggests, the two helix-strand motifs (residues 10–22 and 27–39) are congruent and they can be related to each other by an internal dyad. The dyad also superimposes the two metal ions. The symmetry of the metal-binding domain is readily seen in Fig. 4; a superposition is shown in the accompanying report on the NMR solution structure¹⁸. The only chemically identical residues in each motif are the cysteines, and indeed the six cysteines and two metal ions form most of the core of the domain. The two substructures are joined by a loop that contains a *cis*-proline (residue 26). The proline is conserved in many other homologues of GAL4 (ref. 28). A Pro to Leu mutation at position 26 yields a GAL^- phenotype that can be reversed by high concentrations of Zn^{2+} in the growth medium²⁹. We infer that the substitution at position 26, incompatible with formation of a *cis*-peptide bond, introduces strain in the loop, which can be compensated by higher concentrations of Zn^{2+} .

The recognition module of GAL4 defines a class in the group of DNA-binding domains that have Zn^{2+} as a structural element.

The two other classes for which three-dimensional structures are known are the TFIIIA-like 'zinc-finger'³⁰⁻³² and the steroid-receptor-like DNA-binding module³³⁻³⁵. The only common feature exhibited by these three classes appears to be the use of Zn^{2+} as a crosslink to stabilize a small globular protein.

Dimerization element. Residues 50-64 form an amphipathic α -helix. The dimer interface is formed by the packing of these helices, two of which pack into a parallel coiled-coil (Fig. 2) like those found in α -fibrous proteins³⁶ and in the 'leucine zipper' group of transcription factors^{37,38}. A helical element in this region of the homologous LAC9 protein has been proposed⁶³. The GAL4 fragment in our crystals is monomeric in solution¹⁸ but the contacts made by these helices are sufficiently strong that only dimers are found in the DNA-bound state (R.M., unpublished observation; ref. 7). The interior of the coiled-coil is stabilized by the hydrophobic interaction of three pairs of leucines and a pair of valines, at the *a* and *d* positions of two heptad repeats³⁶. In addition, there are two pairs of Arg-Glu salt links, one between the two chains and one within a helix. The complete GAL4 molecule contains additional residues between 65 and 100 that contribute to dimer interactions and maintain the protein as a dimer even when it is not bound to DNA⁷. The amino-acid sequence of GAL4 is consistent with a coiled-coil that may continue for one heptad repeat beyond the C terminus of GAL4 (1-65). Moreover, residues 79-99 include three potentially α -helical heptad sequences. The intervening segment (residues 72-78) contains a proline. The full dimerization element of GAL4 could therefore share some structural features with the 'helix-loop-helix' transcription factors^{39,40}.

The coiled-coil is positioned over the DNA minor groove, perpendicular to the DNA helix axis. The coiled-coil dyad coincides with the twofold axis of the DNA. The positive ends of the helical dipoles are directed towards the negatively charged sugar-phosphate backbone. The side-chain and main-chain NH of Arg 51, the second helical residue, also contact DNA. A hydrogen-bond network linking Arg 51 to Ser 47 of the other subunit in the dimer helps to maintain the position of the arginine side chain.

Linker region. Residues 41 to 49 are an extended segment connecting the recognition module with the dimerization element (Fig. 3). The path of each linker follows one strand of the DNA backbone. Side chains in the linker are not as well ordered as those elsewhere in the structure. Three of the nine linker residues are positively charged. Lys 43 and Arg 46 lie close to phosphate 4. Lys 45 contacts the phosphate of a symmetry-related DNA molecule in the crystal. The linker terminates with the interaction between Ser 47 and Arg 51 described above, and Pro 48 facilitates the sharp bend required to place the N terminus of the dimerization helix over the DNA minor groove.

DNA structure. The DNA in the complex has only small deviations from a standard B-structure. The average helical twist is 36.0° and the mean rise per base pair is $3.44 \text{ \AA}$. There is a gentle bend ($\sim 7^\circ$) towards the dimerization element. The minor groove is rather wide, as is frequently found in G+C-rich DNA molecules⁴¹, but there is a significant constriction 1 bp to either side of the dyad. This narrowing seems to be imposed by the interaction of phosphates 2, 3, 2' and 3' with the side chains and the main-chain nitrogens of Arg 51 (Fig. 2 and 6). It is therefore possible that these interactions could result in some base sequence preference in the centre of the binding site.

DNA recognition

The consensus GAL4 binding site is a roughly symmetrical, 17-bp element with a highly conserved CCG sequence at either end, reading outwards 5' to 3' from the dyad (Fig. 1c). We have numbered the base pairs sequentially from the centre, with the number 0 assigned to the central base pair. The two recognition modules lie in the major groove separated by about one-and-one-half turns of the DNA helix and centred over the conserved CCG triplets. They are anchored to the sugar-phosphate backbone by hydrogen bonds made by Gln 9 and Arg 15 with phosphate 5, and by Lys 20, Cys 21 (main-chain NH), and Lys 23 with phosphate 6 (Fig. 6). These interactions position the module so that the C-terminal end of the first α -helix points into the groove. The main-chain carbonyl groups of Lys 17 and Lys 18 are exposed at this end of the helix. The carbonyl of Lys 17 accepts a hydrogen bond from the N4 nitrogen of cytosine 8;

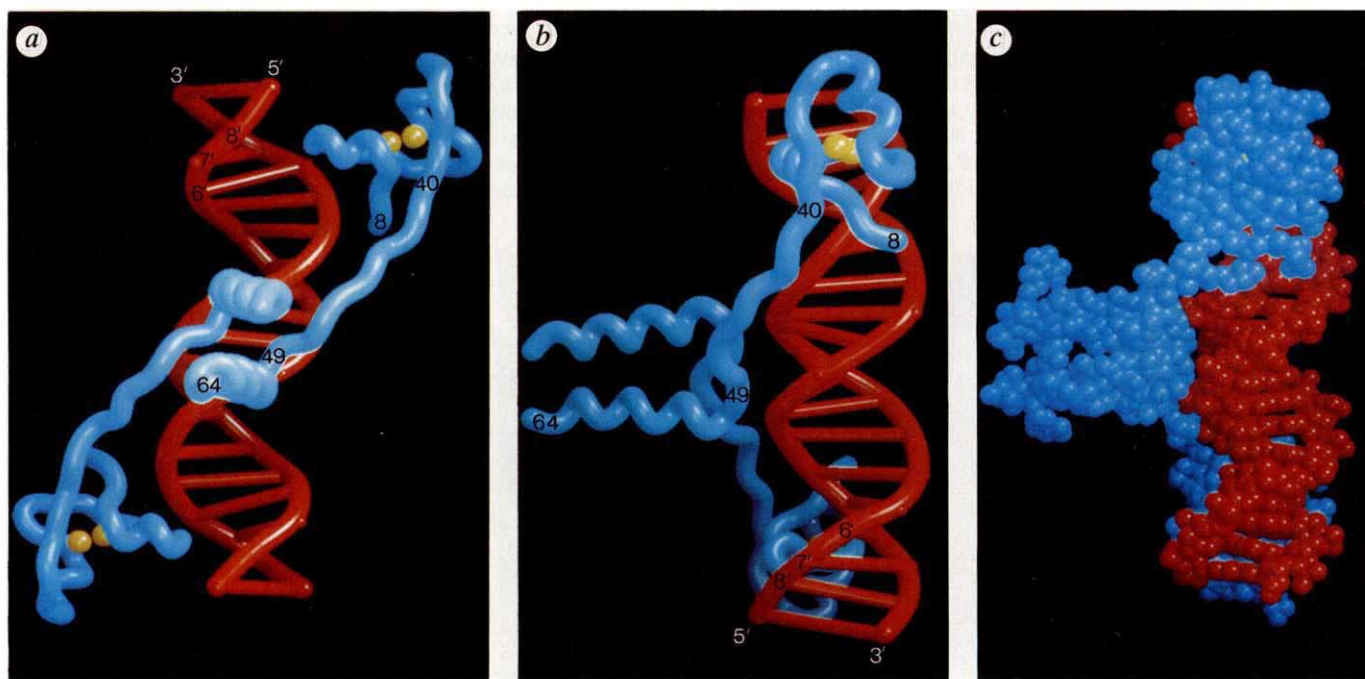


FIG. 3 Structure of the GAL4/DNA complex. *a*, View approximately along the 2-fold axis of the complex. Amino-acid sequence numbers at the borders of the three protein modules (DNA recognition module, residues 8-40; linker region, residues 41-49; and dimerization element, residues 50-64) are

shown on one subunit. DNA (red) is shown as a helical ladder using C3' positions, positions of bound metal ions are shown as yellow balls. *b*, View approximately perpendicular to the 2-fold axis of the complex. *c*, Space-filling model of the complex.

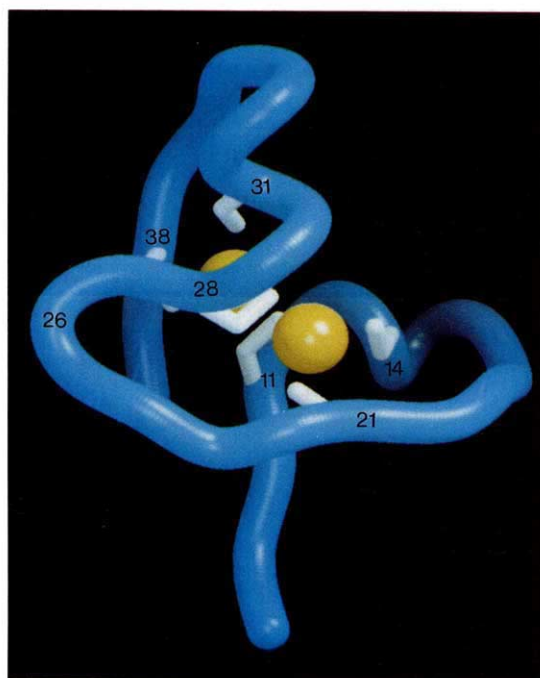


FIG. 4 Structure of the metal-binding DNA recognition module of the protein: C α trace of residues 8–40. The Cys residues that chelate the two metal ions (yellow) are indicated by white sticks. Cysteines and Pro 26 are labelled; cysteines 11, 14, 21 and 28 are the ligands for one metal ion, and 11, 28, 31 and 38, the ligands for the other; residues 11 and 28 are shared by both ions. Amino acids at the C-terminal end of the first of the two helices contact the DNA bases (Fig. 5).

the carbonyl of Lys 18 accepts hydrogen bonds from N4 nitrogens of both cytosine 8 and cytosine 7. The side chain of Lys 18 is in position to donate hydrogen bonds to O6 of guanine 6 and to N7 of guanine 7. This pattern of contacts uniquely specifies the symmetrically conserved CCG sequence at bp 6–8. There are no other direct interactions between protein and DNA base pairs. The model is consistent with binding *in vitro*, using synthetic DNA and GAL4 (1–100), which show that changes in base pairs 6, 7 and 8 have substantial effects on affinity and that changes in the central base pairs 0–5 have relatively little influence (S. Liang, R.M., S.C.H. and M.P., unpublished observation). Specification of two of the three

conserved base pairs by main-chain carbonyls implies that altered specificity cannot be achieved by simple side-chain substitution. The structurally related fungal regulatory proteins LAC9 and PPR1 also recognize a CCG triplet. They could also present two main-chain carbonyl groups to the cytosines of the second and third base pairs, as in the GAL4 complex. Moreover, the homologue of Lys 18 in GAL4 is conserved as lysine in both proteins^{42–44}. Use of a peptide carbonyl to specify a G-C base pair has also been noted in the λ repressor-DNA complex, where the side chain of Lys 3 in the N-terminal arm hydrogen bonds with the N7 of a guanine while the carbonyl of the same residue hydrogen bonds with a cytosine⁴⁵.

The structure formed by residues 47–51 of the two GAL4 subunits at the N-terminal end of the coiled-coil dimerization element is neatly complementary to phosphates 2 and 3 of the two DNA strands (Fig. 2). The dimerization element can therefore be described as a 'minor-groove finder'; its recognition of appropriately presented phosphates probably contributes to specificity as well as to affinity.

The linker that joins the recognition module to the dimerization element interacts with DNA only by salt links between phosphate 4 and the side chains of Lys 43 and Arg 46. These limited DNA contacts, the extended conformation of the linker, and the absence of strong interactions between the linker and the rest of the protein together suggest that the linker could be able to collapse partially without major penalty, permitting specific binding to shorter sites with CGG...CCG sequences. Indeed deletion of the central base pair from a consensus site has <10-fold effect on the *in vitro* affinity of GAL4 (1–100) (S. Liang, R.M., S.C.H. and M.P. unpublished observation) and on the affinity of its homologue, LAC9, for a UAS_G-like site⁴⁶. The observed *in vitro* preference for an 11-bp spacing between CCG triplets shows, however, that an extended linker contributes to a more stable complex.

The structure described here explains why residues both within and C-terminal to the metal-binding domain are important for DNA recognition by GAL4⁶⁴ and its homologue LAC9^{63,65}. The contacts in the structure also account for the results of chemical and enzymatic footprinting experiments⁷. The GAL4 (1–147) fragment protects guanines in base pairs 6 and 7 from methylation, and it protects sugars of nucleotides 0–5 from cleavage by hydroxyl radicals. Ethylation of phosphates 2, 3, 5, 6 and 8' interferes with binding of GAL4 (1–147). All these groups on the DNA are in direct contact with the protein (except phosphate 8', which lies close to Lys 17 but not within range of a salt link in our model). Moreover, we see no close contacts with unprotected groups. The footprinting results

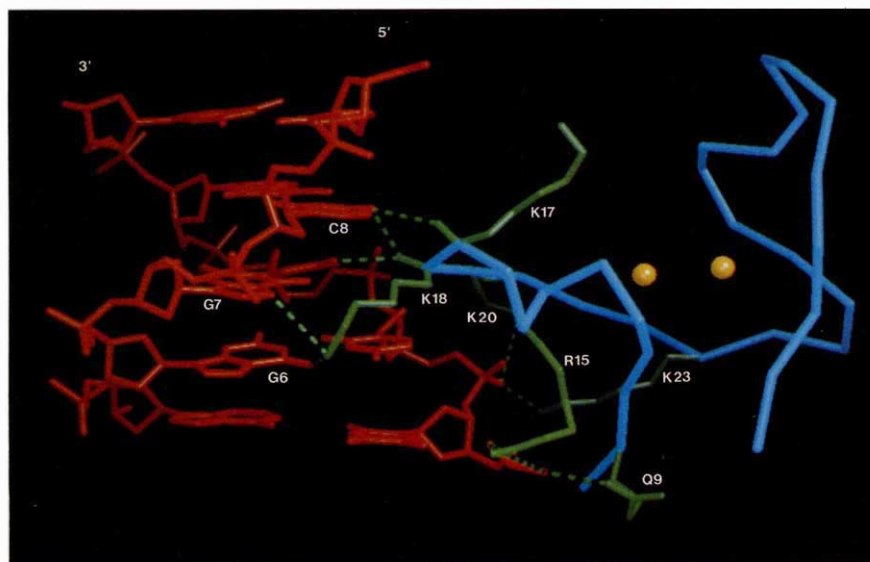


FIG. 5 Protein-DNA contacts mediated by the metal-binding DNA recognition module. For clarity, only the carbon tracing of the protein (blue) is shown, except for the side chains and backbone functional groups (green) that interact with DNA (red). Dashed lines indicate hydrogen bonds between protein and DNA. Lysine 17 could make a hydrogen bond with a phosphate on a longer DNA fragment. Metal ions are indicated by yellow balls. The interaction of the peptide NH of Cys 21 with phosphate 6 is not shown.

led Carey *et al.*⁷ to propose a model with many of the features now seen directly. Moreover, the close correspondence of chemical and crystallographic results rules out significant additional contacts in a GAL4 (1–147) complex not present in the complex with GAL4 (1–65).

In summary, the crystal structure shows that recognition of DNA by a GAL4 dimer combines three components: (1) specificity for the conserved CCG triplets, provided by direct contacts with the recognition modules in the major groove; (2) requirement for a symmetrical site, provided by a coiled-coil dimerization region that lies over the minor groove; and (3) preference for the number of intervening base pairs, provided by the conformation of the linker. It is also possible that there is preference for a particular minor-groove conformation at the centre, provided by the N-terminal end of the coiled-coil element.

Homologous transcriptional activator proteins

At least eleven other fungal DNA-binding proteins are known to contain repeated CX₂CX₆C sequences like those found in the GAL4 recognition module²: LAC9 (ref. 46), PPR1 (ref. 44), QA-1F (ref. 48), QUTA1 (ref. 49), ARGRII (ref. 50), HAP1 (ref. 51), MAL63 (ref. 52), PDR1 (ref. 47), LEU3 (ref. 53), PUT3 (ref. 28), and AMDR (ref. 54). In most of them, the 'loop' between the third and fourth cysteines is six residues long, but it is one residue shorter in MAL63, PDR1, PUT3 and AMDR, and several residues longer in LEU3. GAL4 residues 15–20, which are in closest proximity to DNA in the complex, are highly conserved in these homologues. Arg 15 and Lys 20, which form phosphate salt links that anchor the first helix of the recognition module to DNA (Fig. 5), are conserved in all but AMDR, which has His and Arg at these positions, respectively. Residue 19 is usually hydrophobic, and residue 17 is basic, except in QA-1F and LEU3. Lys 18, which makes base-specific contacts in the GAL4 complex, is conserved in all but three cases. In two of the exceptions (QA-1F and MAL63) it is Arg; in the other (PUT3), it is His. These conservations suggest that the recognition modules of these GAL4 homologues all approach DNA in a similar way. There are symmetrically disposed CCG sequences in known sites for LAC9, PPR1, LEU3 and PUT3. Characteristic heptad sequences suggest that several of the homologue (LAC9, QA-1F, QUTA1, PPR1) contain coiled-coil dimerization elements similar to the one in GAL4. In others such as ARGRII, HAP1, and LEU3, no obvious heptad sequences occur in the 60 residues immediately C-terminal to the recognition modules. In LEU3, the heptads lie one residue closer to the recognition module than in GAL4; in HAP1, they appear to be displaced toward the C terminus by seven residues. Some heterogeneity of dimerization structures and of linker lengths is implied by these observations.

The closest relatives of GAL4 are LAC9, which carries out the same function in *K. lactis*, and PPR1, which regulates

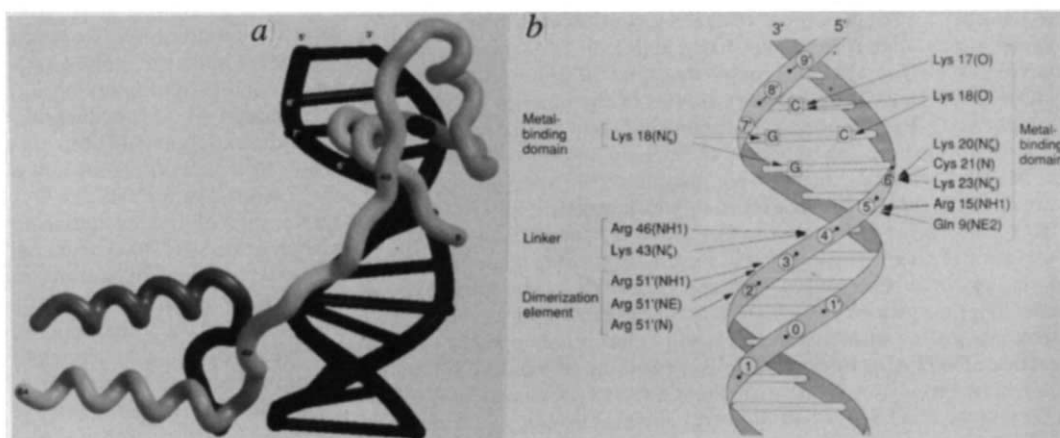
pyrimidine biosynthesis in *S. cerevisiae*. GAL4 and LAC9 bind to the same DNA sites^{42,46}; PPR1 recognizes sites with the CCG triplet separated by six, rather than 11, bp⁵⁵. GAL4 and LAC9 have similar amino-acid sequences in their linker and dimerization segments; the linker and dimerization elements of PPR1 bear no sequence similarity to those of GAL4, aside from the rough characteristics of their heptad regions. Chimaeric molecules containing PPR1 recognition modules and linkers joined to GAL4 dimerization regions bind to PPR1 sites in yeast⁵⁶. These observations demonstrate that in this class of proteins, the characteristics of the linkers specify the spacing between CCG triplets in a binding site. The spacing between DNA half sites is important in other sets of related regulatory proteins, the steroid hormone receptors for example^{57,58}. The GAL4 structure suggests a novel way to achieve specificity for an altered spacing, in the sense that the change involves an extended or flexible linker rather than a modified dimer interface. We note that it is not possible to model a PPR1/DNA complex simply by collapsing the linkers and bringing the recognition modules closer to the centre by three base pairs each, because these modules would collide with the dimerization element. We therefore believe that the coiled-coil in PPR1 must be displaced from the DNA (compared with the GAL4 structure) in order to accommodate the closely spaced recognition modules in the major groove.

Recognition *in vivo*

In the GAL4 (1–65)/DNA complex, the protein wraps around DNA along the minor groove and enters the major groove only at the ends of the site. The 11 central base pairs are accessible to major-groove contacts, either from another protein or from another region of the intact GAL4 molecule. Evidence indicates that the central base pairs form a functional site. Although relatively unimportant for binding of GAL4 fragments *in vitro*, (S. Liang, R.M., S.C.H. and M.P., unpublished observation) the sequences of the central regions are rather well conserved among the known GAL4 sites in yeast (Fig. 1c). Moreover, multiple changes in this region can cause a five- to tenfold decrease in GAL4-mediated transcriptional activity (H. J. Himmelfarb and M.P., unpublished). A possible candidate for the protein that interacts with this site is GAL11. A mutant allele of GAL11, GAL11P, can potentiate transcriptional activation by an otherwise weakly activating derivative of GAL4 (ref. 58). The GAL11P effect is reversed by a mutation at residue 20 in the GAL4 recognition module. Lys 20 is conserved in 10 out of 11 GAL4 homologues (Arg in AMDR). It forms a salt link to phosphate 5, and it is appropriately positioned to interact with a protein that binds in the major groove to exposed central base pairs.

Enhancement of GAL4 specificity by another factor could help account for a problem posed by the observation that only six base pairs are critical for GAL4 affinity. Potential GAL4

FIG. 6 Schematic diagram of protein-DNA interactions in the GAL4 complex. *a*, View chosen is displayed on the left, which shows an α -carbon tracing of the protein (grey), a ladder representation of the DNA (black), and the bound metal ions (black). Relevant parts of the 2-fold-related protein subunit are also shown (dark grey). *b*, Schematic diagram showing all phosphate and base contacts with protein on one side of the 2-fold-related complex. The modular structure of the protein is indicated.



binding sequences should occur far more frequently in yeast regulatory regions than the number of true UAS_G sites. A number of other eukaryotic transcription factors recognize as few as six

base pairs^{35,59}, and evidence suggests that activation *in vivo* normally requires coordinated binding of more than one factor to a complex DNA site⁶⁰⁻⁶². □

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LETTERS TO NATURE

Excitation mechanism of the mesospheric sodium nightglow

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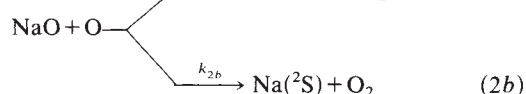
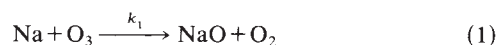
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THE atmospheric sodium nightglow and luminescence of meteor trails, both of which occur at mesospheric altitudes of 85-95 km, are emitted by sodium atoms in the excited 2P state. The Chapman mechanism¹⁻⁸ attributes these to the reaction between NaO and oxygen atoms, requiring an unusually high rate of formation of excited $\text{Na}^*(^2P)$ relative to ground-state $\text{Na}(^2S)$ atoms from the $\text{NaO} + \text{O}$ reaction. But laboratory studies of the kinetics⁹ show a very low $\text{Na}^*(^2P)$ formation rate (branching ratio $f < 0.01$). NaO is itself formed by the reaction of sodium atoms with ozone. Molecular-beam experiments¹⁰ and photoelectron spectroscopy¹¹ have shown recently that this reaction yields largely excited-state ($^2\Sigma^+$) NaO rather than the ground-state ($^2\Pi$) species studied in the $\text{NaO} + \text{O}$ kinetics experiments⁹, thereby suggesting a resolution of the apparent discrepancy with the Chapman mechanism. By extending the symmetry correlation between reactant and product electronic states considered by Bates and Ohja⁶, we show here that reaction of excited-state NaO with oxygen atoms does indeed yield branching ratios consistent with the Chapman mechanism. We infer that the $\text{NaO} + \text{O}$ potential-energy surfaces leading to excited $\text{Na}^*(^2P)$ atoms involve doublet rather than quartet spin configura-

tions, and the branching ratio f is close to zero for ground-state NaO but $\sim 2/3$ for excited-state NaO. If confirmed experimentally, this finding may enable the sodium nightglow to be used as a quantitative measure of mesospheric ozone concentrations^{5,7,8}.

In the reaction sequence proposed in 1939 by Chapman,



the first step is sufficiently exoergic to form NaO in either the $^2\Pi$ or $^2\Sigma^+$ electronic states, with up to ~ 1.8 or 1.6 eV, respectively, in internal or relative translational excitation of the product molecules. In the second step, when excited $\text{Na}^*(^2P)$ atoms are formed the accompanying O_2 molecule must be in its ground electronic state $X^3\Sigma_g^-$, unless the NaO reactant contains roughly 50% of the exoergic of step (1) as vibrational excitation, when the O_2 can also be in its excited $a^1\Delta_g$ state. When ground-state $\text{Na}(^2S)$ atoms are formed, the O_2 can emerge in either the $X^3\Sigma_g^-$ state or one of the excited electronic states $a^1\Delta_g$ or $b^1\Sigma_g^-$, whether or not the NaO reactant is vibrationally excited.

Models for both the sodium nightglow and luminescence of meteor trails require a large value for the product of the rate constant k_1 and the branching ratio $f_2 = k_{2a}/(k_{2a} + k_{2b})$ in order to reconcile the observed intensity of the D-line emission with the known levels of mesospheric Na, O_3 and O, and the Chapman mechanism. A lower limit of

$$k_1 f_2 > 1 \times 10^{-10} \text{ cm}^3 \text{ s}^{-1} \quad (3)$$

was derived by Bates and Ojha⁶, who used data from simultaneous measurements of the nightglow intensity and the mesospheric Na density,^{4,5} coupled with an estimated upper bound for the mesospheric O₃ density. Analysis of more recent data⁷ indicates that the O₃ density is lower by roughly a factor of two, and thus suggests that $k_1 f_2$ is about twice as large as the limit set by equation (3). Recent laboratory kinetic studies^{9,12,13} have found large values for both k_1 and $k_2 = (k_{2a} + k_{2b})$, as expected³ for reactions of alkali species with electrophilic molecules¹⁴. The experimental value¹³, $k_1 = (6.9 \pm 1.5) \times 10^{-10} \text{ cm}^3 \text{ s}^{-1}$ at 216 K, and the limit set by equation (3) imply that the Chapman mechanism requires $f_2 > 0.15 \pm 0.03$. With the amendment by Swider⁷, this corresponds to $f_2 \approx 0.31 \pm 0.09$. The only available experimental estimate⁹ of the branching ratio indicates $f_2 < 0.01$, a discrepancy of at least a factor of 12 and more probably 30. Because the measurements⁹ of k_2 and f_2 were made at atmospheric pressure, however, the NaO reactant is collisionally relaxed, so this discrepancy pertains to the ground electronic state, ${}^2\Pi$.

Now that the primary reaction, step (1), has been found^{10,11} to form NaO largely in the ${}^2\Sigma^+$ state, the key questions in assessing the Chapman mechanism pertain to that state. In condition (3), the rate coefficient k_1 must be replaced by that for forming $\text{NaO}({}^2\Sigma^+)$, which we denote by $k_{1\Sigma}$. The branching ratio f_2 is to be replaced by $f_\Sigma f_\Sigma$, where f_Σ denotes the fraction of $\text{NaO}({}^2\Sigma^+)$ that under mesospheric conditions survives to react with O atoms before being radiatively or collisionally relaxed to $\text{NaO}({}^2\Pi)$, and f_Σ denotes the branching ratio for producing $\text{Na}^*({}^2P)$ atoms from the $\text{NaO}({}^2\Sigma^+) + \text{O}$ reaction. Molecular-beam magnetic-deflection analysis¹⁰ indicates that $k_{1\Sigma}$ is between

$\sim 1.0k_1$ and $0.8k_1$. For f_Σ and f_Σ , we must at present use theoretical estimates. The survival factor f_Σ is probably near unity. Radiative decay of NaO by ${}^2\Sigma^+ \rightarrow {}^2\Pi$ is negligibly slow¹⁵ compared with its expected rate of reaction with mesospheric O atoms. At the peak nightglow altitude of 88 km, the O atom density has been measured^{16,17} as 0.7×10^{11} to $5.0 \times 10^{11} \text{ cm}^{-3}$; thus, assuming that $\text{NaO}({}^2\Sigma^+)$ reacts with roughly the same rate measured⁹ for $\text{NaO}({}^2\Pi)$ of $3.7 \times 10^{-10} \text{ cm}^3 \text{ s}^{-1}$, the chemical lifetime of $\text{NaO}({}^2\Sigma^+)$ would be less than 0.04 s at this altitude. Electronic quenching of $\text{NaO}({}^2\Sigma^+)$ by mesospheric species other than O atoms (primarily N₂ and O₂) is also expected to be quite slow, although O atoms constitute only 0.1–1% of the total number density^{16,17}. Moreover, in $\text{NaO}({}^2\Sigma^+) + \text{O}$ collisions, reaction is expected to be much more probable than unreactive quenching. The branching ratio f_Σ is thus the main question.

Figure 1 presents a correlation diagram for the triatomic Na–O–O potential energy surfaces that connect the electronic states of the reactants and products for Chapman's step (2). As usual¹⁸, the surfaces and states are classified by the total electronic spin degeneracy, which is either doublet or quartet, and by the spatial symmetry (A' or A'') of the electronic wavefunction with respect to the plane of the three atoms, the only symmetry element preserved in the reaction. Electronic structure calculations¹⁹ for NaO show that both the ${}^2\Pi$ and ${}^2\Sigma^+$ states are strongly ionic (Na^+O^-), so the reactions with O involve an electron transfer. We thus assume that the ion-pair states arising from $\text{NaO}^+ + \text{O}^-$ are traversed, so only surfaces linking reactant and product states through these ion-pair states contribute to reaction.

The lower portion of Fig. 1, pertaining to $\text{NaO}({}^2\Pi) + \text{O}$, is equivalent to the symmetry analysis provided by Bates and Ojha⁶. The reactants give rise to 12 potential surfaces, three belonging to each of the four symmetry species. Only six of these correlate with the ion-pair states, three doublet and three quartet states. In turn, the three doublet states and one of the quartet states correlate with $\text{Na}({}^2S) + \text{O}_2$ (in the energetically accessible states, X, a and b); the other two quartet states correlate with $\text{Na}^*({}^2P) + \text{O}_2$. This corresponds to $f_\Pi = 1/3$, as suggested by Bates and Ojha. But because the quartet states involve three electrons with parallel spins, we expect the corresponding potential surfaces to be highly repulsive and unlikely to permit reaction. If reaction occurs only by way of doublet surfaces, $f_\Pi = 0$, consistent with the experimental upper limit (< 0.01) on the branching ratio obtained by Plane and Husain⁹.

The upper portion of Fig. 1 shows the analogous analysis for $\text{NaO}({}^2\Sigma^+) + \text{O}$. Here the reactants give rise to six surfaces, and all correlate with the ion-pair states, which have higher electronic degeneracy and therefore could accommodate 12 surfaces. Again, we expect the quartet states to be too repulsive to contribute. One of the doublet states correlates with the sole state of $\text{Na}({}^2S) + \text{O}_2$ not connected to the $\text{NaO}({}^2\Pi) + \text{O}$ manifold; the other two doublet states correlate with $\text{Na}^*({}^2P) + \text{O}_2$. This predicts $f_\Sigma = 2/3$, a value large enough to satisfy condition (3).

In Fig. 1, unlike Bates and Ojha, we have not included correlations with $\text{Na}^+ + \text{O}_2^-$ ion-pair states. These do not introduce relevant constraints. The lowest $2A'$ and $2A''$ surfaces, linking $\text{NaO}({}^2\Pi) + \text{O}$ with $\text{Na} + \text{O}_2$, correlate with $\text{Na}^+({}^1S) + \text{O}_2^-({}^2\Pi_g)$. Excited states of the O_2^- ion²⁰ are omitted because these are diffuse resonances and lie at fairly high energies, hence cannot contribute appreciably to the wavefunctions for the reaction surfaces. As the ionic reaction complex dissociates, the itinerant electron seldom resides equally on the two oxygen atoms, except perhaps for the lowest $2A'$ and $2A''$ surfaces.

Vibrational excitation of the NaO reactant could make $\text{Na}^* + \text{O}_2(a^1\Delta_g)$ energetically accessible. According to Fig. 1, however, this would not affect the branching ratio unless quartet states do contribute; all the doublet states stemming from $\text{NaO}({}^2\Pi) + \text{O}$ lead to less-excited product channels.

The actual magnitude of the branching ratio depends not only on the number of linking surfaces but also on their shape and

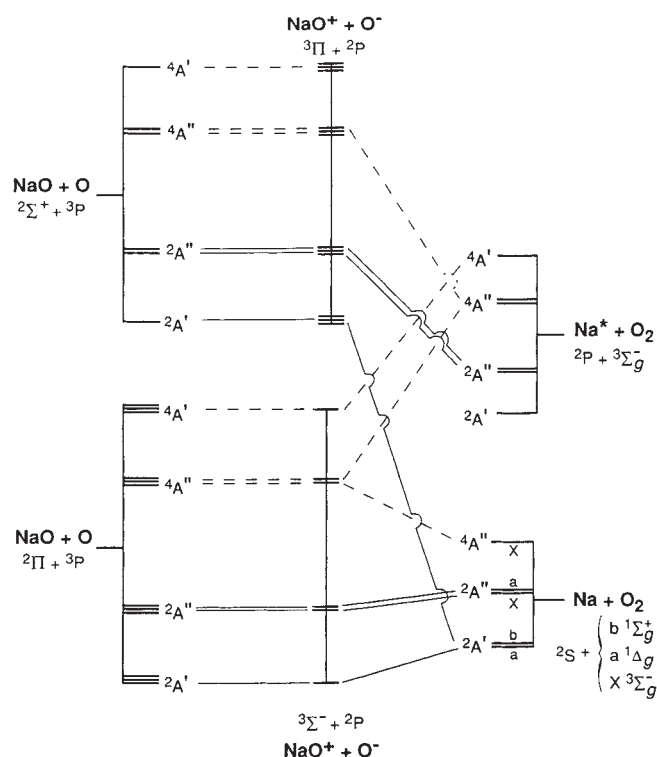


FIG. 1 Correlation of electronic states of reactants, kindred ion-pairs and products for step (2) of the Chapman mechanism. The four distinct types of states react separately, because the electronic symmetry species and total spin do not change during reaction. For states of symmetry species A' the wavefunction does not change sign on reflection in the plane of the three atoms; for species A'' it does change sign. Full lines show correlations of states with total electronic spin of $1/2$ (doublets), dashed lines those with spin $3/2$ (quartets). Potential surfaces arising from the quartet states are likely to be too repulsive to permit reaction.

location. Our analysis simply serves to show how f_{Π} can be very small but f_{Σ} large. For instance, two surfaces of the same symmetry repel each other, so only the lower one of the pair of $^2A''$ surfaces in Fig. 1 that correlate with $Na^+ + O_2$ might be accessible; the branching ratio would then be reduced to $f_{\Sigma} = 1/2$.

Another aspect of the correlation analysis deserves emphasis. In Fig. 1, the reaction surfaces arising from $NaO(X^2\Pi)$ are correlated through those for $NaO^+(X^3\Sigma^-)$ and the surfaces from $NaO(A^2\Sigma^+)$ through those for $NaO^+(A^3\Pi)$. Yet electronic structure calculations and photoelectron spectroscopy¹¹ for NaO^+ show that near the equilibrium bond distance, the $X^3\Sigma^-$ and $A^3\Pi$ states differ in energy by only 0.3–0.5 eV. In the electron transfer process, NaO in either the $^2\Pi$ or $^2\Sigma^+$ state thus might be expected to form NaO^+ almost equally well in both the $^3\Sigma^-$ and $^3\Pi$ states. The correlation analysis would then become much less restrictive and could not account for a small value of the branching ratio for the $NaO(^2\Pi) + O$ reaction (for example f_{Π} would then be 1/3 for doublet states). The analysis of Fig. 1 assumes that the electron transfer just involves one of the valence electrons least involved in bonding. In terms of the dominant molecular orbital configurations, this corresponds to



and



Such a criterion, widely exemplified in organic chemistry²¹, often overrides energetic considerations²².

Our analysis seems to reconcile the Chapman mechanism with both laboratory results and mesospheric data. But we advocate experiments to test whether f_{Π} is indeed very low but f_{Σ} high, and to measure rates for the reaction steps involving the $^2\Sigma^+$ state of NaO . As well as finally establishing the Chapman mechanism, this would enable aeronomers to determine mesospheric ozone levels from the sodium nightglow emission and simultaneous lidar measurements of the sodium profiles^{5,7}. This indirect procedure would be welcome because it is difficult to measure upper mesospheric ozone directly²³. We also advocate electronic structure calculations to test our correlation diagram. The selectivity inferred from Fig. 1 does not stem from symmetry considerations alone. Mediation by ion-pair states⁶ has a key role, as well as the postulates that only doublet states react and that the itinerant electron is least involved in bonding. These criteria, if confirmed, should be applicable to a host of metal oxidation reactions capable of producing electronically excited states. □

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Structural and electronic properties of sodium-intercalated C_{60}

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ALKALI metal fullerenes exhibit superconductivity^{1–4} at temperatures surpassed only by the copper-oxide-based ceramics. Three types of stoichiometry and structure have been identified: A_3C_{60} (ref. 5) (where A is K or Rb) with A^+ cations intercalated in the face-centred cubic (f.c.c.) C_{60} structure, and A_6C_{60} (ref. 6) or A_4C_{60} (ref. 7) (where A is K, Rb or Cs) with body-centred arrays of C_{60} molecules. All A_3C_{60} compounds reported so far are superconductors with transition temperatures that increase with unit cell size^{8,9}. No successful preparation of bulk Na_xC_{60} has been reported, although measurements of electrical conductivity¹⁰ and Raman¹¹, ESR¹¹ and photoemission¹² spectra of thin films have given clear indications of Na_xC_{60} formation. Here we report the synthesis and initial characterization of bulk Na_xC_{60} ($2 \leq x \leq 6$) and mixed alkali phases Na_2AC_{60} (where A is K, Rb, or Cs). All of these phases have intercalated f.c.c. structures. The Na_6C_{60} structure has a Na_4 cluster centred on the octahedral site. The Na_2AC_{60} compounds superconduct for the larger A cations, but a crossover to nonsuperconducting behaviour occurs with decreasing cation size and correlates with a minimum in the unit cell volume.

Direct reaction of C_{60} with alkali-metal vapour in sealed pyrex tubes has been the predominant synthetic route to A_xC_{60} (where A is K, Rb or Cs), but the lower vapour pressure and greater reactivity with pyrex are a severe problem for reactions with sodium. Therefore, we have explored alternative preparative routes and found that sodium metal in stainless steel vessels, and sodium-containing compounds such as NaN_3 , NaH , $NaBH_4$ and Na_2Hg_2 , can be effective reagents for synthesis of Na_xC_{60} . We synthesized the saturated composition, Na_6C_{60} , by reaction of C_{60} and excess Na in a sealed stainless steel tube

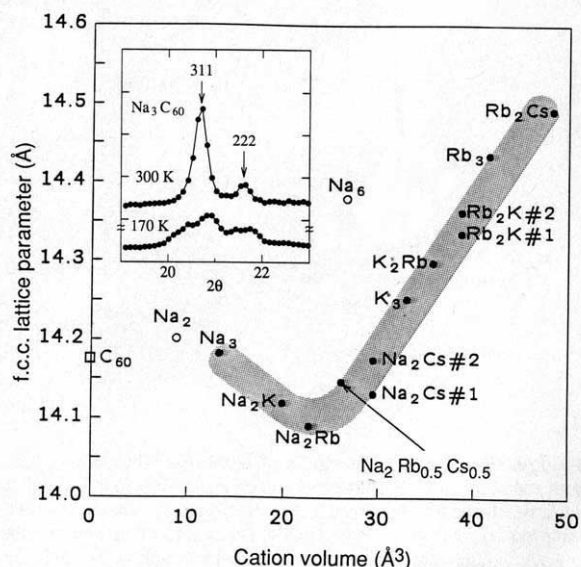


FIG. 1 The f.c.c. lattice parameter of A_3C_{60} as a function of total volume of the intercalated cations. Insets: powder X-ray diffraction patterns of Na_3C_{60} at 300 K and 170 K.

TABLE 1 X-ray powder data for Na_xC_{60} .

h	k	l	$\text{Na}_2\text{C}_{60}^*$		$\text{Na}_3\text{C}_{60}^\dagger$		$\text{Na}_6\text{C}_{60}^\ddagger$	
			I_0	I_c	I_0	I_c	I_0	I_c
1	1	1	917	1,000	320	650	260	252
2	0	0	51	85	0	21	0	16
2	2	0	564	608	214	378	366	341
3	1	1	1,000	893	1,000	1,000	1,000	1,000
2	2	2	257	339	217	205	393	339
4	0	0	0	14	0	22	0	9
3	3	1	75	98	0	32	76	31
4	2	0	27	32	0	52	0	12
4	2	2	280	359	252	288	308	165
5	1	1	99	160	60	64	316	208
3	3	3	99	160	60	64	316	208
4	4	0	36	40	33	32	108	18
5	3	1	0	2	0	1	0	3
4	4	2	0	18	0	2	50	36
6	0	0	0	18	0	2	50	36
6	2	0	0	0	0	1	0	4
5	3	3	0	25	31	17	44	18
6	2	2	62	68	36	18	81	58
4	4	4	0	3	0	0	0	19
5	5	1	26	48	26	26	56	28
7	1	1	26	48	26	26	56	28
6	4	0	51	50	25	11	62	0
6	4	2	0	2	0	0	0	25

Data are calculated for $Fm\bar{3}m$ (subcell of p.c. Na_2C_{60}). Zeros represent unobserved reflections.

* 8 Na at (c) $43m$ ($\frac{1}{2}, \frac{1}{2}, \frac{1}{2}$); 4 C_{60} at (a) $m\bar{3}m$ (0,0,0); $R_F=15.7\%$, $R_w=13.5\%$ using all reflections including those unobserved; $T=300\text{ K}$; $a=14.189(1)$. ((a), (c) and so on are the Wyckoff positions of the sites.)

† 8 Na at (c) $43m$ ($\frac{1}{2}, \frac{1}{2}, \frac{1}{2}$); 4 Na at (b) $m\bar{3}m$ ($\frac{1}{2}, \frac{1}{2}, \frac{1}{2}$); 4 C_{60} at (a) $m\bar{3}m$ (0,0,0); $R_F=10.3\%$, $R_w=9.7\%$ using all reflections including those unobserved; $T=300\text{ K}$; $a=14.191(4)$.

‡ 8 Na at (c) $43m$ ($\frac{1}{2}, \frac{1}{2}, \frac{1}{2}$); 32 Na with occupancy 0.5(1) at (f). $3m$ (x, x, x), $x=0.430(6)$; 4 C_{60} at (a) $m\bar{3}m$ (0,0,0); $R_F=14.5\%$, $R_w=12.0\%$ using all reflections including those unobserved, $T=10\text{ K}$; $a=14.248(8)$ ($a=14.381(8)$ at 300 K).

heated in an argon flow (350 °C for 4 hours, 400 °C for 72 hours, 350 °C for 6 days), then opened and excess sodium distilled away from the product. Na_2C_{60} was synthesized by grinding stoichiometric amounts of NaH and C_{60} together in a dry box and heating to 350 °C in a sealed pyrex tube for nine days with two intermediate regrindings. Other reagents were used in a similar manner. Na_3C_{60} was prepared most cleanly by reaction of Na_5Hg_2 with C_{60} (250 °C for 7 days, then 350 °C for 3 days) followed by distillation of mercury away from the product. Ternary $\text{Na}_2\text{AC}_{60}$ phases were synthesized by reaction of NaH, NaN_3 or Na_5Hg_2 with A_6C_{60} (where A is K, Rb or Cs) and C_{60} at 250 °C for 24 hours followed by several days at 330 °C.

The X-ray powder diffraction patterns of all the Na_xC_{60} and $\text{Na}_2\text{AC}_{60}$ compositions prepared can be indexed readily on f.c.c. unit cells. Table 1 summarizes the observed powder diffraction data for Na_xC_{60} and the calculated intensities for models presented below. The lattice constants for all the materials are plotted in Fig. 1 as a function of the total cation volume (calculated from tabulated ionic radii). Least-squares refinements of the integrated intensities of the non-degenerate reflections in $Fm\bar{3}m$, using a spherical-shell form factor for C_{60} , indicate that Na occupies primarily the tetrahedral sites in Na_2C_{60} ($R_w=13.5\%$). The restricted angular range of the data results in strong correlation between the scale and temperature factors, which are consequently unphysical and not quoted. Careful examination of the high-angle region of the X-ray pattern reveals weak reflections violating the f.c.c. extinctions but satisfying primitive cubic (p.c.) symmetry. The intensities of the additional reflections are qualitatively similar to the low-temperature $\text{Pa}\bar{3}$ orientationally ordered structure of pristine C_{60} (ref. 13).

The sodium-saturated (Na_6C_{60}) phase has a f.c.c. X-ray pattern with a considerably larger lattice constant ($a=14.380\text{ Å}$) than for $x=2-3$. The octahedral site (4b at $\frac{1}{2}, \frac{1}{2}, \frac{1}{2}$, $r=2.06\text{ Å}$) is a poor size match for Na^+ ($r=1.02\text{ Å}$). We therefore considered a model (Fig. 2) with displacements along the 3-fold $\langle 111 \rangle$ axes of the f.c.c. cell which create a cubic cluster of 8 sites on the 32f x, x, x positions, and allow for a sodium concentration greater than 3 in the f.c.c. phase. A similar arrangement has recently been suggested¹⁴ for Ca_xC_{60} . The integrated intensity refinement in $Fm\bar{3}m$ with Na^+ cations on both the 32f and tetrahedral 8c sites at $\frac{1}{4}, \frac{1}{4}, \frac{1}{4}$ proved extremely sensitive to both the position and occupancy of the 32f sites, converging to $x=0.435$, $n=0.4$ at 300 K and $x=0.430$, $n=0.5$ at 10 K ($a=14.248$), corresponding to an approximate composition Na_6C_{60} (addition of water followed by titration of liberated base gives a composition $\text{Na}_{5.9\pm 0.3}\text{C}_{60}$). No lowering of symmetry was observed at 10 K. Inspection of F_{obs} Fourier scattering density

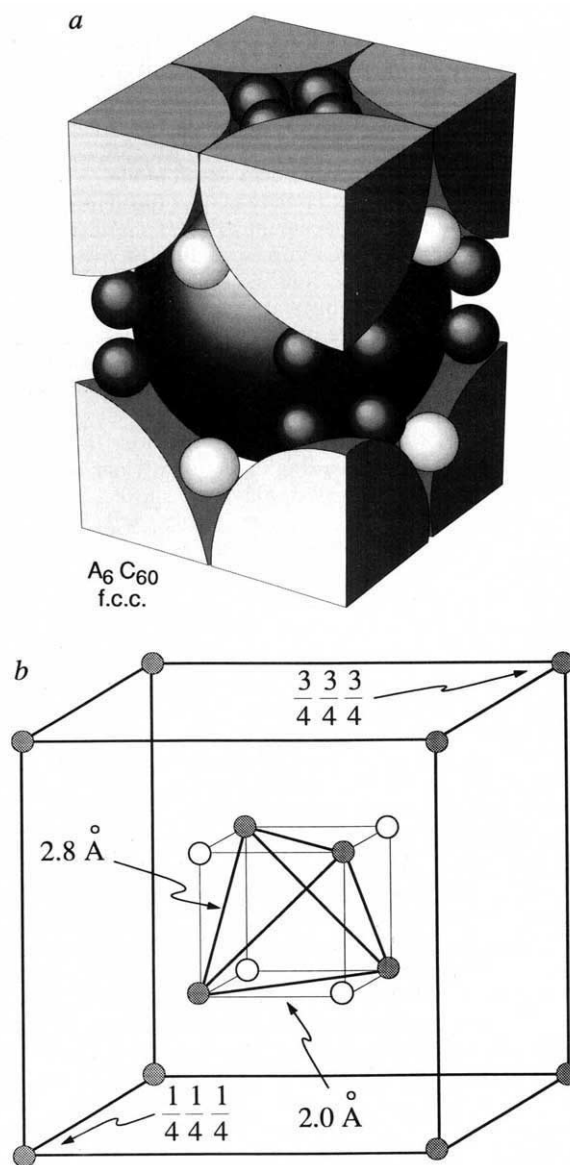


FIG. 2 Crystal structure of Na_6C_{60} . a, body-centred tetragonal representation of the f.c.c. cell of A_6C_{60} . The larger spheres represent C_{60} and the smaller spheres Na. The darker Na sites are half occupied in Na_6C_{60} . b, Sodium sites: the corners of the large cube are the tetrahedral sites and the smaller central cube shows the two disordered orientations of Na_4 tetrahedra (one orientation is shaded) centred on the octahedral site. The C_{60} molecules forming the octahedral site lie above the centre of each face of the larger cube.

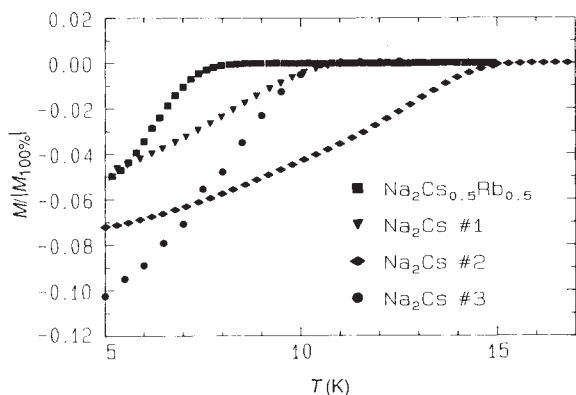


FIG. 3 Zero-field-cooled d.c. magnetization of superconducting $\text{Na}_2(\text{Cs}, \text{Rb})\text{C}_{60}$ ($H=5\text{ Oe}$). The Meissner effect (field-cooling diamagnetism) is typically 10–20% of the zero-field-cooled diamagnetic signal.

maps confirmed the model used. Consideration of the Na^+-Na^+ distances within the cube, $2.0\text{ \AA}$ along an edge and $2.8\text{ \AA}$ across a face diagonal, suggests that the refinement corresponds to a disordered distribution of the two possible orientations of Na_4 tetrahedra, as indicated in Fig. 2 (right). Refinement of an ordered arrangement of the tetrahedra in the non-centric $F43m$ gives no improvement over the disordered model, with residual electron density in the F_{obs} Fourier map at the centrosymmetric equivalent positions. Replacement of the spherical-shell form factor by C atom positions corresponding to two disordered orientations of C_{60} with 2-fold axes along $[100]$, as used in K_3C_{60} (ref. 5), gave a small but statistically insignificant improvement in the R factors. The presence of Na_4 clusters raises the issue of covalent Na–Na interactions which might lead to incomplete charge transfer from Na to C_{60} . Differences between the Raman spectra of Na_xC_{60} and those of other A_xC_{60} compounds suggested partial charge transfer¹¹. In addition, the ^{13}C NMR spectrum of Na_6C_{60} shows a single, motionally narrowed resonance at 167 p.p.m., which is significantly shifted from that of Rb_6C_{60} (154 p.p.m.)¹⁵.

The structural phase diagram of Na_xC_{60} for $0 \leq x \leq 6$ differs from that of the heavier alkalis by allowing $0 < x < 6$ within the f.c.c. structure. Therefore, the $x=3$ composition which gives rise to superconductivity when A is K or Rb does not necessarily correspond to a line phase. Extensive regions of solid solution are possible, although the nonlinearity of a with x suggests the existence of intermediate phases or ordering. The structure of a sample with nominal composition Na_3C_{60} (elemental analysis 9.05% Na; calculated for Na_3C_{60} 8.73%) refines well for Na occupation of both tetrahedral and octahedral sites ($R_w=9.7\%$), although because of the large thermal parameters we cannot exclude positional disorder of the octahedral Na (for example on the 32f sites) or small deviations from $x=3$.

All A_3C_{60} compounds previously reported have been superconducting. Our previous correlation of T_c with the unit cell size of A_3C_{60} (ref. 8) and data on the pressure dependence of T_c (refs 17, 18) and a (ref. 19) suggest that the T_c of Na_3C_{60} should be near 16 K for the observed lattice parameter. We observe no superconductivity, however, above 2 K in Na_3C_{60} . Significantly, we find that below 250 K there is a change in the X-ray pattern (insert to Fig. 1) which seems to result from

disproportionation into two f.c.c. phases with lattice parameters close to those of Na_2C_{60} and Na_6C_{60} .

Refinements of the X-ray powder intensities of $\text{Na}_2\text{AC}_{60}$ (where A is K, Rb or Cs) indicate ordering of Cs^+ and Rb^+ cations on the octahedral site (when A is Rb, $R_{\text{ordered}}=11.7\%$, $R_{\text{disordered}}=33.5\%$), whereas in $\text{Na}_2\text{KC}_{60}$ the ordered model ($R=14.1\%$) is only a slight improvement over the disordered ($R=19.7\%$). The unit cell size of A_3C_{60} at room temperature has a minimum as a function of total cation volume near $\text{Na}_2\text{RbC}_{60}$ (Fig. 1). We attribute the unexpectedly large cells of the branch with low cation volume to Na occupancy of the octahedral site. This further implies displacement of Na from the centre of the site. $\text{Na}_2\text{KC}_{60}$ undergoes a disproportionation near 250 K qualitatively similar to that of Na_3C_{60} , whereas $\text{Na}_2\text{RbC}_{60}$ remains f.c.c. at 10 K.

Of the ternary $\text{Na}_2\text{AC}_{60}$ compounds, only $\text{Na}_2\text{CsC}_{60}$ and $\text{Na}_2\text{Rb}_{0.5}\text{Cs}_{0.5}\text{C}_{60}$ are superconducting above 2 K (Fig. 4). All the superconducting samples, including three different $\text{Na}_2\text{CsC}_{60}$ samples (nominal compositions) with different T_c , have lattice parameters consistent with the nearly linear dependence of T_c on a shown by other A_3C_{60} compounds. The unit cell of $\text{Na}_2\text{CsC}_{60}$ no. 1 ($a=14.132\text{ \AA}$, $T_c=10\text{ K}$) is smaller than that of C_{60} itself ($a=14.17\text{ \AA}$) and close to the cell size and T_c for K_3C_{60} under 7 kbar pressure^{17–19}. The value of $\tau_1 T$ for $\text{Na}_2\text{CsC}_{60}$ ($T_c=10\text{ K}$) is constant between 150 K and 100 K ($\tau_1 T=190 \pm 15\text{ K s}$), consistent with the reduced T_c arising from a lower density of states at E_F (17 states per eV per C_{60} per spin, using the approximations in ref. 16) than in K_3C_{60} . (T_1 is the ^{13}C NMR spin-lattice relaxation time, and $T_1 T$ is constant for a metal²⁰.)

The absence of superconductivity above 2 K in $\text{Na}_2\text{RbC}_{60}$, which has a lattice parameter close to that of K_3C_{60} under 10 kbar pressure ($T_c \approx 12.5\text{ K}$)^{17–19}, indicates that the chemical influence of Na^+ is more pronounced than a simple size effect. The value of $\tau_1 T$ increases by a factor of two (from 99 to 205 K s) from 298 K to 103 K, leaving open the question of whether $\text{Na}_2\text{RbC}_{60}$ is metallic. The crossover from superconducting to nonsuperconducting behaviour coincides with the minimum in the room-temperature unit cell size as a function of cation volume (Fig. 1) and the onset of disproportionation at low temperature. □

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Superconductivity in sodium- and lithium-containing alkali-metal fullerides

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THE discovery¹ of superconductivity, with a transition temperature T_c of 18 K, in potassium-doped C_{60} was followed by the synthesis of other superconducting M_3C_{60} phases: Rb_3C_{60} ($T_c = 28$ K and 30 K; refs 2, 3), $CsRb_2C_{60}$ ($T_c = 31$ K; ref. 4) and Cs_2RbC_{60} ($T_c = 33$ K; ref. 4). A monotonic relationship is observed⁵ between T_c and lattice constant a_0 for these face-centred cubic M_3C_0 compounds, all of which have a_0 values larger than that of pure C_{60} . Here we study this relationship over a wider range using mixed alkali compounds incorporating sodium (Na_2MC_{60} , where M is K, Rb or Cs) and lithium (Li_2CsC_{60}). The Na_2MC_{60} compounds have face-centred cubic structures and are superconducting, with $T_c = 12$ K (M = Cs), 2.5 K (M = Rb) and 2.5 K (M = K); these T_c s are lower than those predicted on the basis of high-pressure studies⁶⁻⁸. This departure from the previous relationship between T_c and a_0 for lattice parameters smaller than that of pristine C_{60} may provide new insights into what controls superconductivity in these materials.

We purified the original crude mixture of C_{60}/C_{70} (Texas Fullerene) in a neutral aluminium column using a hexane/toluene elutant followed by heat treatment under 1×10^{-6} torr pressure. First the alloys Na_2M and Li_2M were made

TABLE 1 Transition temperatures and lattice parameters of f.c.c. M_3C_{60} superconductors

Material	T_c (K)	a_0 (Å)	Fraction (%)
$RbCs_2C_{60}$	33	14.555 (0.007)	60
Rb_2CsC_{60}	31	14.431 (0.006)	60
Rb_3C_{60}	29	14.384 (0.010)	70
KRb_2C_{60}	27	14.323 (0.010)	84
K_2CsC_{60}	24	14.292 (0.010)	60
K_2RbC_{60}	23	14.243 (0.010)	75
K_3C_{60}	19	14.240 (0.006)	70
Na_2CsC_{60}	12	14.134 (0.006)	72
Li_2CsC_{60} *	12	14.120 (0.021)	1
Na_2RbC_{60}	2.5	14.028 (0.011)	2
Na_2KC_{60}	2.5	14.025 (0.021)	0.1
$Na_2CsC_{60}^\dagger$	12		36
$Na_2CsC_{60}^\ddagger$	12		8
C_{60}		14.161 (0.009)	

* Fraction of f.c.c. phase from the X-ray diffraction pattern is much less than that for other compositions at room temperature, probably because of difficulty in preparation.

† Prepared from the nominal composition $Na_{1.5}Cs_{1.5}C_{60}$.

‡ Prepared from the nominal composition $NaCs_2C_{60}$.

in sealed quartz tubes (5 mm in diameter) by heating at $\sim 150^\circ\text{C}$ under helium atmosphere. The C_{60} was then introduced into the prepared alloys in a glove box. Other samples were prepared as reported elsewhere⁴. The quartz tubes containing C_{60} and the dopant alloy were degassed to 10^{-2} torr and refilled with helium to 700 torr twice before sealing. These were heated in a furnace at 200°C for 24 hours and then at 420°C for 72 hours. We used a SQUID magnetometer (Quantum design MPMS) to measure the temperature dependence of the d.c. magnetization of the samples. We first cooled the samples to 2 K under zero magnetic field (ZFC mode), then applied a magnetic field of 10 Oe and measured the magnetic shielding as a function of temperature up to 50 K. Magnetic flux expulsion (the Meissner effect) was observed when the samples were cooled in a magnetic field of 10 Oe (FC mode). The same samples were transferred into thin capillary tubes for X-ray analysis to determine the crystal structures and lattice constants.

X-ray diffraction patterns of the prepared M_3C_{60} compounds with nominal compositions Na_2CsC_{60} , Na_2RbC_{60} and Na_2KC_{60} are shown in Fig. 1 together with Rb_3C_{60} as a reference. For all compositions, the predominant phase is f.c.c. and other phases are negligibly small. The calculated f.c.c. lattice constants are

$$\begin{aligned} a_0(Rb_3C_{60}) &= 14.384 \text{ Å} > a_0(K_3C_{60}) = 14.240 \text{ Å} > a_0(C_{60}) \\ &= 14.161 \text{ Å} > a_0(Na_2CsC_{60}) \\ &= 14.134 \text{ Å} > a_0(Na_2RbC_{60}) \\ &= 14.028 \text{ Å} > a_0(Na_2KC_{60}) = 14.025 \text{ Å}. \end{aligned}$$

These values follow the trend expected from the alkali-metal ionic radii, and the lattice constants for Na_2MC_{60} are smaller than pristine C_{60} , whereas those for all previous doped phases have been larger⁵. It seems likely that the superconducting phase would be ordered f.c.c. Na_2MC_{60} with the larger M atom in the octahedral site and the smaller Na atoms in the two tetrahedral sites. Refinements of the X-ray diffraction intensities of Na_2MC_{60} indicate that Cs and Rb cations are indeed preferentially located in the octahedral sites ($\chi^2_{ord} = 0.86$, $R_{ord} = 10.5$ and $\chi^2_{dis} = 18.7$, $R_{dis} = 43.7$ for Na_2CsC_{60} ; $\chi^2_{ord} = 1.18$, $R_{ord} = 11.3$ and $\chi^2_{dis} = 22.4$, $R_{dis} = 46.2$ for Na_2RbC_{60} (ref. 9), where 'ord' and 'dis' refer to the ordered and disordered states). In the case of Na_2KC_{60} , we were unable to determine the degree of ordering in the crystal.

We measured the transition temperatures of this series of binary-metal-doped C_{60} compounds using a SQUID and found that Na_2CsC_{60} and Li_2CsC_{60} become superconducting at $T_c = 12$ K, and Na_2RbC_{60} and Na_2KC_{60} at $T_c = 2.5$ K, as shown in

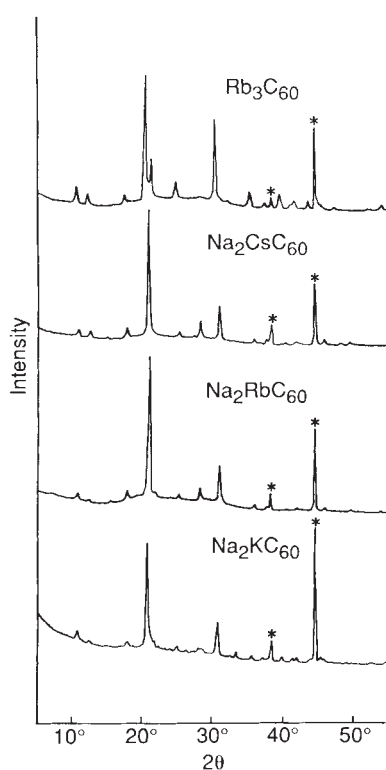


FIG. 1 X-ray diffraction patterns for Rb_3C_{60} , Na_2RbC_{60} and Na_2KC_{60} . All samples show f.c.c. crystal phase (lattice constants are listed in Table 1). Peaks indicated by asterisks are due to background noise from the aluminium sample holder.

Table 1. Typical curves of susceptibility against temperature for the nominal compositions $\text{Na}_2\text{CsC}_{60}$ and $\text{Na}_2\text{RbC}_{60}$ are shown in Fig. 2. The measurements were done in both ZFC and FC modes. Magnetic expulsion and shielding are clearly observed in both modes, with onset temperatures of 12 K and 2.5 K respectively. The observed T_c 's unambiguously indicate that the superconducting phases obtained for these nominal compositions result from the ternary $\text{Na}_2\text{MC}_{60}$ (where M is K, Rb or Cs) and $\text{Li}_2\text{CsC}_{60}$ compounds, as C_{60} doped with the individual elements does not become superconducting in the cases of Cs_xC_{60} and Na_xC_{60} (refs 3, 5, 10), and furthermore, $T_c = 18$ K for K_3C_{60} (ref. 1) and 28–30 K for Rb_3C_{60} (refs 2, 3). We have also confirmed that Cs_xC_{60} ($x = 1$ to 6) and Na_xC_{60} ($x = 1$ to 6) are not superconducting. Preliminary experiments on changing the compositions of $\text{Na}_x\text{Cs}_y\text{C}_{60}$ ($x + y = 3$) further support the idea that $\text{Na}_2\text{CsC}_{60}$ is the only possible superconducting mixture. A T_c of 12 K is always observed regardless of the nominal compositions, and the fraction of the superconducting phase becomes maximum at $x = 2$ and $y = 1$, as shown in Table 1.

For the purpose of discussion, we show in Fig. 3 three sets of data relating T_c to the lattice dimension: the experimental relationship between T_c and a_0 from pressure studies, theoretical predictions, based on $N(E_F)$, made by local density approximation (LDA) calculations, and the experimental T_c 's for various f.c.c. M_3C_{60} compounds.

The pressure dependence of T_c for K_3C_{60} (ref. 6) and Rb_3C_{60} (ref. 7) demonstrates that T_c decreases with pressure (P), and shows that a parabolic-type curve can connect T_c - P data from Rb_3C_{60} ($T_c = 29$ K, 0 kbar) to highly pressurized K_3C_{60} ($T_c = 8$ K, 32 kbar). The plots of T_c as a function of a_0 in Fig. 3 were taken from ref. 8. We also show a plot of T_c against a_0 obtained from the calculated relationship between $N(E_F)$ and a_0 (ref. 10), supposing a phonon-mediated simple BCS equation $T_c \sim \omega_{ph} \exp[-1/VN(E_F)]$ if the phonon frequency ω_{ph} and the phonon-electron coupling constant, V , are assumed to vary negligibly with a_0 , as discussed elsewhere⁵. The dotted line in Fig. 3 expresses this relationship obtained for $V = 17$ meV and $\omega_{ph} = 133.3 \text{ cm}^{-1}$ (these values were estimated from $N(E_F)$ values¹¹ in combination with the T_c 's of K_3C_{60} ($T_c = 19$ K) and Rb_3C_{60} ($T_c = 29$ K)). This value of V is close to the reported theoretical calculation¹² of $V = 40$ MeV implying relatively strong phonon-electron coupling, whereas the resulting ω_{ph} is very sensitive to the data used for the density of states and varies

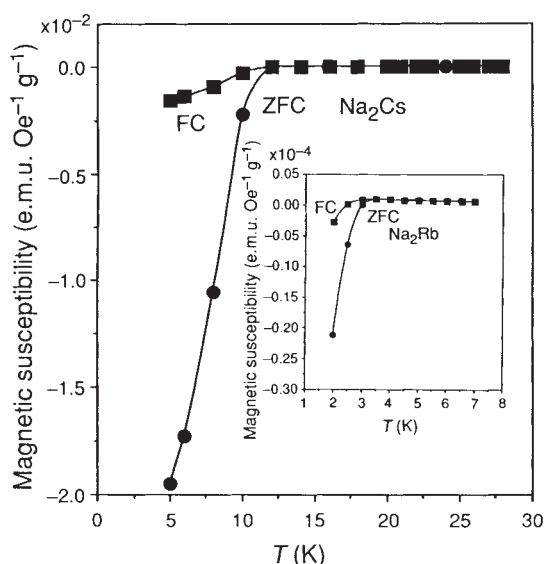


FIG. 2 Magnetic susceptibilities of $\text{Na}_2\text{CsC}_{60}$ and $\text{Na}_2\text{RbC}_{60}$, field-cooled and zero-field-cooled.

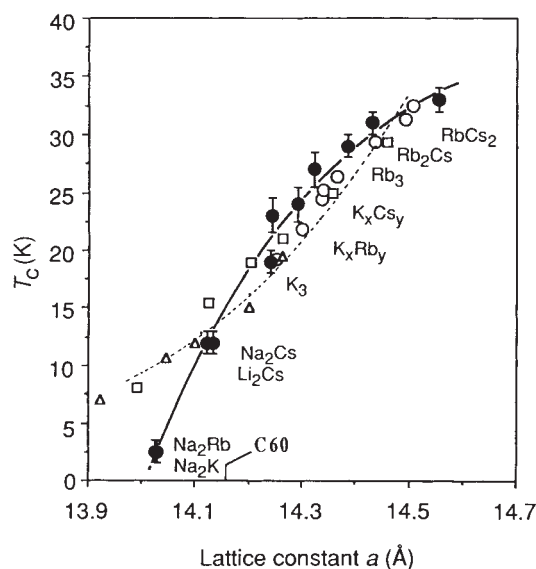


FIG. 3 The relationship between T_c and a_0 for M_3C_{60} superconductors (where M is Li, Na, K, Rb, Cs and their binary alloys) over a wide range of a_0 . Data indicated by open circles are taken from ref. 5; our experimental data (closed circles) are listed in Table 1. The solid line is curve fitted to both sets of data. The open triangles and squares are T_c - a_0 relationships for K_3C_{60} and Rb_3C_{60} obtained from pressure experiments⁸, and the dotted line is the T_c - a_0 relationship predicted from LDA calculations¹¹ of $N(E_F)$ values, as described in the text.

from intra-phonon to inter-phonon modes (for example, values of $N(E_F)$ from NMR spectroscopy¹³ give $V = 11$ meV and $\omega_{ph} = 1,410 \text{ cm}^{-1}$).

The pressure and $N(E_F)$ plots in Fig. 3 agree fairly well with the observed relationship between T_c and a_0 for M_3C_{60} compounds of compositions ranging from $\text{Rb}_2\text{CsC}_{60}$ ($T_c = 31$ K, $a_0 = 14.431 \text{ Å}$) down to roughly $\text{Na}_2\text{CsC}_{60}$ ($T_c = 12$ K, $a_0 = 14.134 \text{ Å}$); but for smaller lattice parameters, the pressure and $N(E_F)$ plots deviate significantly from the new experimental data. The experimental curve appears convex instead of concave. This difference comes as a surprise, as one of the most reasonable suggestions so far for the origin of superconductivity in f.c.c. M_3C_{60} has been electron pairing mediated by high-frequency C_{60} intramolecular or low-frequency intermolecular phonon modes^{2,5,14,15}, both of which expected to vary negligibly with the observed changes in lattice parameters. A simple monotonic universal relationship between T_c and a_0 might still be true although the curvature is different from that previously assumed. The fact that we find the same T_c for different dopants with the same lattice constants (for example $\text{M}_2\text{CsC}_{60}$ where M is Na or Li, and $\text{Na}_2\text{MC}_{60}$ where M is Rb or K) supports this interpretation. But the systematic reduction in superconducting fraction on going from $\text{Na}_2\text{CsC}_{60}$ (72%) to $\text{Na}_2\text{Rb}_1\text{C}_{60}$ (2%) to $\text{Na}_2\text{K}_1\text{C}_{60}$ (0.1%), despite almost the similar amount of f.c.c. phase observed at room temperature, suggests that the material is approaching a boundary between a superconducting f.c.c. phase and another, nonsuperconducting phase, as suggested by Rosseinsky *et al.*¹⁶. The differences between the pressure data and our results cannot easily be explained in this way unless some other parameter (other than a_0) is also changing in this region.

Stepniak *et al.*¹⁷ pointed out from photoemission results that C_{60} doped with small alkali ions (for example, Li or Na) are on the verge of a metal-insulator transition¹⁷. We should also remark here that the most stable crystal phase of Na_xC_{60} (made by direct reaction of C_{60} with Na) is not f.c.c., but seems likely from our preliminary X-ray analyses to be body-centred tetragonal or another type of crystal. We may therefore attribute the observed superconducting phase of $\text{Na}_2\text{MC}_{60}$ (M = K, Rb)

to a metastable superconducting phase existing near the phase boundary between the superconducting f.c.c. and non-superconducting phase. In this boundary phase, $N(E_F)$ may decrease markedly or phonons mediating electron pairing may be strongly affected, leading to a different relationship between the observed T_c and a_0 . If that is the case, a universal monotonic relationship does not exist between T_c and lattice constant, and the experimental curve in Fig. 3 is then actually composed of two distinct areas, one corresponding to the pure f.c.c. superconducting phase and the other to a metastable phase in the small a_0 region.

Lithium, sodium and caesium-doped C_{60} do not show any

superconducting properties^{3,5,10}. In the case of Cs-doped C_{60} , this has been explained by the fact that the structure is body-centred cubic^{5,10}. For Li- and Na-doped C_{60} no explanation has yet emerged for the lack of superconductivity. Considering the trend observed for the mixed-alkali Na and Li superconductors reported here, we might speculate that, like Na_xC_{60} (ref. 16), Li_xC_{60} has no stable f.c.c. superconducting phase. If, however, metastable superconducting phases exist for these compounds, they would show T_c s around 2.5 K, as the lattice would not contract any further on going from Na_2RbC_{60} ($a_0 = 14.028 \text{ \AA}$) or Na_2KC_{60} ($a_0 = 14.025 \text{ \AA}$) to Na_3C_{60} . □

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Importance of methane-oxidizing bacteria in the methane budget as revealed by the use of a specific inhibitor

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METHANE is a greenhouse gas whose concentration in the atmosphere is increasing^{1–3}. Much of this methane is derived from the metabolism of methane-generating (methanogenic) bacteria^{4,5}, and over the past two decades much has been learned about the ecology of methanogens; specific inhibitors of methanogenesis, such as 2-bromoethanesulphonic acid, have proved useful in this regard⁶. In contrast, although much is known about the biochemistry of methane-oxidizing (methanotrophic) bacteria⁷, ecological investigations have been hampered by the lack of an analogous specific inhibitor⁶. Methanotrophs limit the flux of methane to the atmosphere from sediments^{8,9} and consume atmospheric methane¹⁰, but the quantitative importance of methanotrophy in the global methane budget is not well known⁵. Methylfluoride (CH_3F) is known to inhibit oxygen consumption by *Methylococcus capsulatus*¹¹, and to inhibit the oxidation of $^{14}CH_4$ to $^{14}CO_2$ by endosymbionts in mussel gill tissues¹². Here we report that methylfluoride (MF) inhibits the oxidation of methane by methane monooxygenase, and by using methylfluoride in field investigations, we find that methanotrophic bacteria can consume more than 90% of the methane potentially available.

We demonstrated MF inhibition of methane monooxygenase with samples from a seasonally dry lakebed (Table 1). The ability of these soils to consume methane and to oxidize ^{14}C -methane to ^{14}C -carbon dioxide was effectively blocked by addition of 1.6% MF to the headspace; inhibition also occurred with as little as 0.01% MF. Similar results were obtained with peaty soils from a different location (Sacramento River Delta), in which 1.6% MF blocked methane consumption over prolonged time periods (>200 h) with no loss of MF from the headspace (data not shown). In experiments with only 0.16% MF, however, we did observe loss of both methane and MF after ~120 h incubation. The inhibition by MF was specific for methane monooxygenase and did not affect methanol dehydrogenase or formate dehydrogenase. We incubated triplicate sets of peat

soils (5 g, for 120 h at 20 °C) wetted with 3 ml deionized water in sealed serum bottles (air gas phase 47 ml) with either 2.0 μCi of ^{14}C -methanol (59 mCi mmol⁻¹; Amersham Corporation) or 2.5 μCi of ^{14}C -sodium formate (50 mCi mmol⁻¹; ICN Company). After acidification (2.5 ml of 6M HCl) and 12 h shaking, $^{14}CO_2$ was quantified¹³. Soils incubated with 6.7% by volume MF formed $0.85 \pm 0.01 \mu Ci$ and $1.02 \pm 0.11 \mu Ci$ (mean $\pm$ 1 s.d.) of $^{14}CO_2$ from the ^{14}C -methanol and the ^{14}C -formate, respectively. Controls incubated without MF formed 0.85 ± 0.08 and

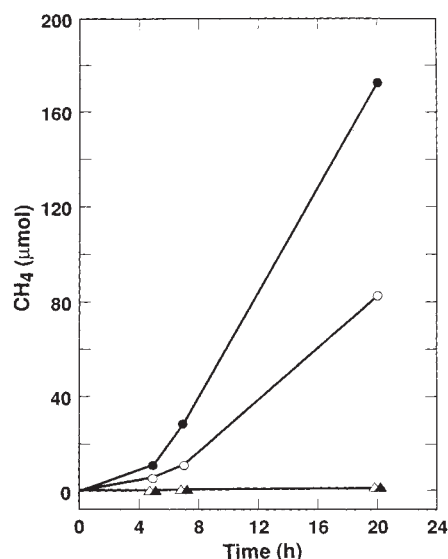


FIG. 1 Accumulation of methane in the headspace of duplicate sets of chambers incubated on the upslope region of the Searsville Lake shoreline on 9 October 1991. Chambers incubated with 40-ml additions of MF (○, ●); control chambers without MF (Δ, ▲). Purity of MF was $\geq 99\%$ (Matheson Gas Company). Cylindrical plexiglass chambers (diameter 19 cm, area 284 cm², gas volume 4 l, modified by removal of the handpump as described elsewhere¹⁷) were inserted into the sediment to a depth of ~3 cm. The chambers' eight sampling ports were sealed with serum stoppers, with the exception of two ports in which balloons (volume ≈ 20 ml) were attached to prevent thermal pressure differentials. Subsamples (5 ml) were withdrawn periodically by syringe, stored by displacement of water in small sealed test tubes and analysed by flame-ionization gas chromatography, usually within 1 h of collection¹⁸.

TABLE 1 Inhibition of methane oxidation in Searsville Lake sediments

0 h		48 h		123 h	
Control	With MF	Control	With MF	Control	With MF
¹⁴ C-methane in headspace (μCi)					
0.55	0.55	0.14	0.59	0.00	0.72
(0.02)	(0.02)	(0.03)	(0.01)	—	(0.02)
Methane in headspace (μmol)					
28.6	28.6	6.9	30.6	0.00	46.2
(1.0)	(0.2)	(1.8)	(0.3)	—	(9.8)
¹⁴ C-carbon dioxide in headspace (μCi)					
0.0	0.0	0.04	0.0	0.28	0.0
—	—	(0.004)	—	(0.007)	—
Carbon dioxide in headspace (μmol)					
2.2	2.2	21.1	18.1	1,594	1,577
(0.2)	(0.2)	(1.1)	(1.3)	(63)	(109)

Results represent the mean (1 s.d.) of triplicate 57-ml serum bottles (gas-phase volume was 32 ml) containing 30 g of sediment, ~40 μmol of added methane, 0.5 ml MF, ~1.0 μCi of ¹⁴C-methane (Amersham Corporation; specific activity 56 mCi mmol⁻¹; purity 97.5%) incubated statically at 20 °C. Some adsorption (~25%) of both methane and ¹⁴C-methane to the sediment surfaces occurred during the incubation. Gases were analysed by gas chromatography coupled with gas proportional counting for determination of radiocarbon¹³. For extraction of dissolved CO₂, samples were acidified with 7 ml of 10 M HCl, 12 h before analysis. Results at 123 h represent values obtained after acid extraction.

1.21 ± 0.13 μCi of ¹⁴CO₂ respectively. Because there were no significant differences, the above results indicate the specificity of MF, and the fact that it is applicable to diverse soil or sediment types.

We demonstrated inhibition of methane oxidation by MF with cell suspensions of *M. capsulatus* grown overnight to turbidity ($A_{680} = 0.42$). Cells were grown by the method described in ref. 14, modified by supplementation of the medium with 1.2 mg L⁻¹ CuSO₄. Cells were dispensed (16 ml) into conical flasks (57 ml volume) and sealed under air with 1.2% CH₄ with or without 1.2% MF, and incubated at 34 °C with constant shaking (150 revolutions per minute). Consumption of methane by controls was linear for more than 4 h at a rate of ~3.2 μmol h⁻¹. Cells inhibited with MF did not consume methane, but slowly consumed the MF (~0.3 μmol h⁻¹). A suspension which had demonstrated a methane oxidation rate of 3.3 μmol h⁻¹ for 2 h was injected with 1.2% MF. There was no further loss of methane or MF from this flask over the subsequent incubation period (2.25 h). These results indicate that methanotrophs slowly oxidize MF, explaining its disappearance from soil samples over prolonged incubations (see above).

MF may have ancillary inhibitory effects on other physiological groups of bacteria⁶, most notably the methanogens. We incubated anoxic sediment slurries from a saltmarsh¹⁵ with and without addition of 10% MF to the headspace of 57-ml serum bottles containing 20 ml of sediment slurry (1:1) sealed under N₂. After 167 h, triplicate controls produced 9.3 ± 0.9 nmol CH₄ per ml slurry (mean ± 1 s.d.) whereas those with MF formed 5.7 ± 0.3 nmol CH₄ per ml slurry, equivalent to 39% inhibition. By contrast, slurries incubated with an equivalent amount of acetylene, a potent inhibitor of methanogenesis¹⁶, were inhibited by 96% (0.39 ± 0.05 nmol CH₄ per ml slurry). In a second experiment in which sulphate was excluded from the slurry salts¹⁵, controls produced 548 ± 15 nmol CH₄ per ml slurry after 183 h, whereas those with 10% and 1.25% MF formed 152 ± 9.6 and 350 ± 24.7 nmol CH₄ per ml slurry, equivalent to 72 and 36% inhibition, respectively. Evidently methanogens in sulphate-free sediments were more sensitive to MF than those with sulphate. But the inhibition in both systems was only partial. The efficacy of MF inhibition of methanotrophs at even lower concentrations (for example at 0.01% by volume) suggests that the concentration of MF can be adjusted downward to an empirically derived value (~0.1–1.0% by volume) that inhibits methane oxidation without disrupting methanogenesis.

To test this, we needed material with an active methanogenic and methanotrophic flora. Adding MF to such material should result in enhanced accumulation of methane when incubated under an air atmosphere. We collected shredded plant compost from the Palo Alto refuse disposal area. Treatment for the compost consists of watering and turning, conditions which promote anaerobiosis and aerobiosis, respectively. Compost was dispensed (~700 ml) into 962-ml glass jars and sealed under air. Rubber septa in the jars' metal caps permitted syringe sampling of the gas phases. Methylfluoride (1 ml; ~0.4% of gas phase) was added to three jars, and three controls received no MF addition. The material was shaken by hand and, after initial sampling, allowed to incubate at ~20 °C. After 21 h incubation, controls formed 1.5 ± 1.7 μmol CH₄ per l compost, whereas those with MF formed 12.4 ± 2.8 μmol CH₄ per l compost (mean ± 1 s.d.). These results indicated that MF selectively inhibited methanotrophs, but not methanogens.

We next did field experiments with chambers to determine whether MF addition would enhance methane flux. Initial experiments were done on a seasonally exposed sandbar on the perimeter of Searsville Lake, California. Addition of 1% by volume MF to the headspace of replicate chambers resulted in the steady accumulation of more methane than in uninhibited controls. For example, in one experiment after 24.5 h incubation there was a ~100-fold difference between the average methane concentrations in the inhibited and control chambers (Fig. 1). Over this same time interval, MF in the headspace of the inhibited chambers declined by 61% (data not shown). Immediately after the chambers were removed, short cores were taken from the central axis of the chambers and extracted for methane and MF. Inhibited chambers had higher methane concentrations in the upper 9 cm of these sediments than did the controls (Fig. 2). Additionally, MF was present at all depths from cores taken under the inhibited chambers, but was not detected in cores from nearby controls (~10 cm away). These results indicate that

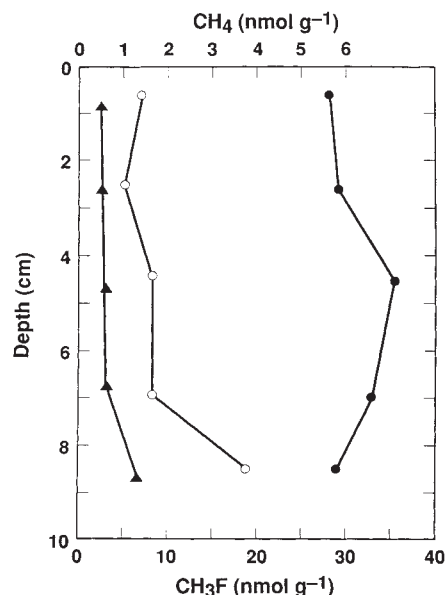


FIG. 2 Profiles of methane and MF in subcores collected after 24.5-h chamber incubation from Searsville Lake on 9 October 1991. ○, Methane and ●, MF under an inhibited chamber. ▲, Methane under a control chamber (MF was not detected). Cores fashioned from 60-ml plastic syringes (hub-end removed) were used to sample the upper ~9 cm of sediment, which consisted of fine silt mixed with sand layers. The cores were stoppered and were extracted within 3 h of collection. Subsections of cores were extruded into wide-mouth flasks (250 ml) containing 150 ml of NaCl-saturated water. The flasks were sealed and shaken for 1 h, and the gas phases analysed for methane and MF by flame-ionization gas chromatography¹⁷.

TABLE 2 Methane flux determined by *in situ* chamber incubation

	Methane flux ($\mu\text{mol m}^{-2} \text{h}^{-1}$)	
	Without MF	With MF
Searsville Lake		
8 October, shoreline, 22.3 h	58.7	405.8
	279.0	2,653.8
9 October, upslope, 24.5 h	0.6	124.0
	1.2	248.5
10 October, upslope, 24.5 h	0.3	191.8
	1.5	26.9
21 October, upslope, 5.8 h	2.9	85.6
	25.1	104.0
22 October, upslope, 27 h	1.2	6.0
	2.9	66.6
San Francisco Bay saltmarsh		
25 October, 5.3 h	2.0	-0.5
	5.0	3.8
31 October, 23.3 h	2.6	2.5
	7.4	7.9
La Honda Creek		
5 November, creek sediments, 23 h	-0.3	3.6
	0.1	9.4*

All dates are 1991.

* Leak detected after second sampling; rate extrapolated for 4.25 h incubation.

the decline in MF observed with time in the inhibited chambers was due to its downward flux into the unsaturated sediments, and that lateral transport was impeded by the high solubility of MF (~1.7 ml MF per ml water) in the underlying pore water. Furthermore, MF seems to have changed the abundance and shape of the methane profile beneath the chambers by eliminating its oxidation and allowing for a greater flux out of the sediment. The degree of loss of MF from the chambers seemed to be related to the water content of the substrate. In chambers deployed on water-saturated saltmarsh sediment (see below), only a minor loss ($\leq 10\%$) occurred over 24 h. On permeable moist soil or compost, however, more than 90% of the MF added to chambers was lost within 4 h (not shown).

The results of chamber deployments in three different environments are given in Table 2. For each of the five incubations conducted at Searsville Lake, addition of MF always resulted in a greater methane flux than in control chambers. The enhancement of methane flux by MF varied from a minimum of threefold to over 400-fold. The absolute magnitude of the fluxes measured on any day varied, as did the response to MF addition. This suggests that the methane content and the extent of methanotrophic activity in the sediment around the incubation site (~100 m²) varied considerably. Similar variability in methanotrophic activity has been observed previously⁹. Nonetheless, the fact that there was consistently higher flux with MF indicates that the inhibition was effective. Addition of MF had no effect on methane release for the two incubations conducted at the saltmarsh site. These tidally flooded sediments are highly reducing¹⁵ and the lack of oxygen at this site probably precluded significant methanotrophy. The third site consisted of an exposed, wet freshwater streambed located in the riparian region of La Honda Creek. At this site the enhancement of methane flux by MF was obvious.

Our results indicate that when methanotrophy is present in soils, it restricts the amount of methane released. More than 90% was consumed at some sites. Similar estimates have been made indirectly for rice paddies, either by comparing anaerobic incubations (which could enhance methanogenesis) with aerobic ones¹⁹, or by calculating oxidation as the difference between methanogenic activity and outward flux²⁰. Use of MF in field investigations should overcome the ambiguities inherent in indirect assessments of methane oxidation. □

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Molecular record of secular sea surface temperature changes on 100-year timescales for glacial terminations I, II and IV

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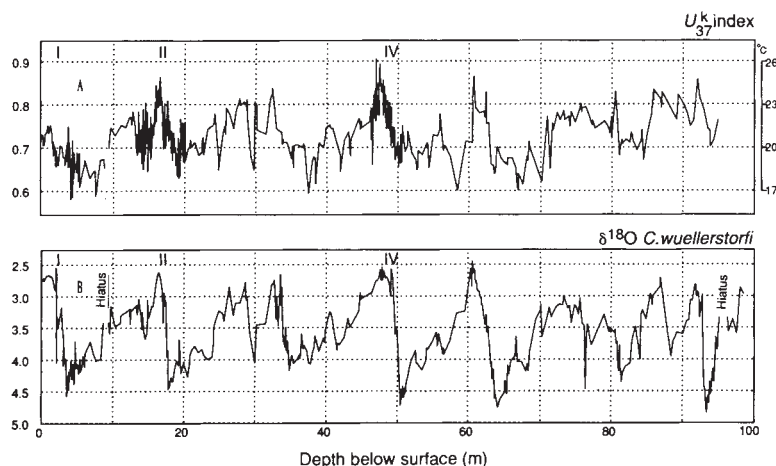
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HIGH-RESOLUTION palaeoclimate records based on oxygen isotope data have provided important insights into climate variability and rates of natural climate change on about a thousand-year timescale¹⁻³. Little is known⁴, however, about the variation of climate and of palaeoceanographic conditions throughout the Quaternary on timescales of less than 1,000 years (at frequencies far greater than those of the Milankovitch orbital cycles). Here we show that such high time resolution is possible from molecular stratigraphic studies based on 'biomarker' organic molecules (alkenones). We have sampled alkenone stratigraphic records at 70- to 200-yr intervals across glacial terminations I, II and IV in sediment cores from ODP site 658, off northwest Africa⁵. Sea surface temperatures (SSTs) derived from the alkenone (U_{37}^k) index⁶⁻⁸ vary rapidly beyond the range of analytical noise by up to 2.5 °C in 300 yr, showing hitherto unknown cycles with about 600-yr periodicities. Some of the changes parallel similar events in the oxygen isotope stratigraphy. SST oscillations may be linked, in part, to abrupt breakdowns in Atlantic deep-water ventilation resulting from meltwater events of Quaternary glacial terminations^{3,9,10}.

ODP site 658 was cored⁵ below the high-productivity zone of the main upwelling cell off northwest Africa, on the continental slope 160 km west of Cape Blanc¹¹ (20° 45' N, 18° 35' W; 2,263 m water depth). The coastal upwelling off Cape Blanc is driven by the northeast trade winds on a perennial basis, producing cold nutrient-rich surface waters with modern temperatures as low as 16 °C. On the basis of pre-site surveys of bottom topography, we selected the site to give a largely undisturbed hemipelagic palaeoenvironmental record, providing a continuous 100-m thick sediment section spanning the Brunhes chron^{3,5,12}. The high average sedimentation rate of ~15-20 cm per 1,000 yr

FIG. 1 Down-hole stratigraphies for (a) alkenone unsaturation- U_{37}^k and (b) $\delta^{18}O$ for *C. wuellerstorfi*³, for ODP holes 108–658 A and B. The extent of the data, down to 100 m below surface (mbsf) composite depth, represents the past 730 kyr of the sedimentary record. The temperature scale is based on the calibration of Prah and Wakeham⁸. Glacial terminations I, II and IV are represented by core sections which have been sampled at closely spaced intervals (1 cm or more) for the U_{37}^k analysis. Each sample was taken with a 1-cm-diameter tube.



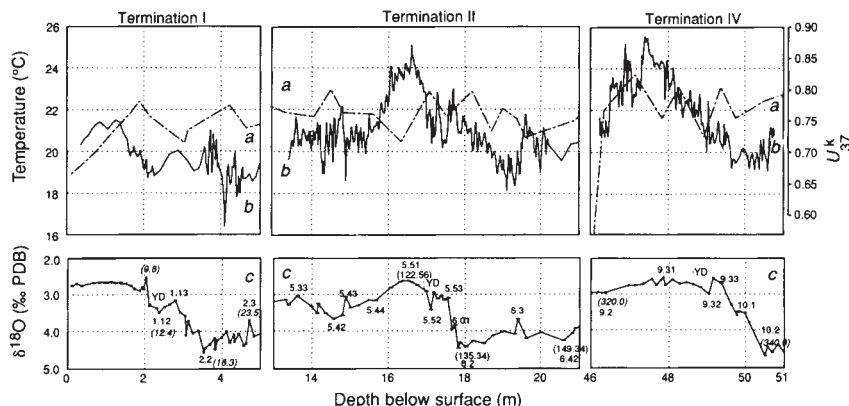
provided a suitable opportunity to sample organic-rich sediment (0.5–2.0% total organic carbon) with narrow spacings of 1- and 2-cm intervals. Each sample was 1 cm in diameter, corresponding to ~50–100 yr of sedimentation, and the sample spacing corresponded to a resolution of 75–200 yr (ref. 3). We are aware that this range of data spacing is subject to a considerable smoothing of signals related to the average bioturbational mixing depth, estimated^{13–15} to reach 10–15 cm, but the signal from this 1-cm sample spacing is nevertheless better extracted than from slit sampling, of say, 10 cm of core.

Molecular analyses for alkenones can be based on sediment samples an order of magnitude smaller (100 mg) than those needed for the evaluation of most other palaeoenvironmental proxy data such as foraminiferal transfer functions¹⁶ and oxygen isotopes^{3,17}. Moreover, molecular analyses are independent of calcium carbonate dissolution, which can strongly perturb the other proxy measures¹⁸. Finally, laboratory automation permits the processing of large numbers of samples. Empirical calibration of the U_{37}^k index through rather limited measurements of cultures, water-column particulate material⁸ and core tops¹⁹ indicates that a shift of 0.1 in U_{37}^k corresponds to a change of ~3 °C in SST. In absolute numbers, $U_{37}^k = 0.75$ equates with 21.4 °C, the error of the analytical method being ± 0.8 °C with 68% probability (A.R., manuscript in preparation). The U_{37}^k data were smoothed to minimize the analytical error in the graphical representation. The average absolute values of the difference between raw U_{37}^k and smoothed curve is 40% of the analytical error, with a negligible effect on climatological signals.

We compare the alkenone-based SSTs with summer SSTs

derived from planktonic foraminiferal assemblages²⁰ using the Modern Analogue technique²¹ in a new version for the Atlantic (U.P. *et al*, manuscript in preparation). In this technique, SSTs derived from a transfer function record the temperature regime of the ocean surface layer for roughly the upper 150 m down to below the thermocline, and the summer and winter seasons are assessed separately. Hence, the transfer-function-based SSTs cannot be compared precisely with alkenone-based SSTs of the surface layer (upper ~10 m). Summer and winter transfer-function curves are very similar, with the winter values ~4 °C colder throughout. We also compare U_{37}^k data with isotopic $\delta^{18}O$ data for benthic (*Cibicides wuellerstorfi*) foraminifera^{3,17} used for climatic stratigraphy. The full set of raw U_{37}^k data and the $\delta^{18}O$ data for benthic foraminifera are presented in Fig. 1. Except for the temperature of the present warm stage, most features of the benthic $\delta^{18}O$ oscillations are reflected in the SST- U_{37}^k signal over the past 650,000 yr in the Milankovitch frequency range, as to be expected from previous work^{7,8,18}. The very-high-resolution sampling afforded a separate presentation of the data in Fig. 2 which focuses on glacial terminations I, II and IV, encompassing their associated climate optima and subsequent cooling phases. The raw U_{37}^k data showed numerous oscillations of ~1–3 °C over 200–300 yr, but after smoothing (Fig. 2), changes of up to 2.5 °C in periods of ~300 yr are still apparent. We cannot assess the true magnitude of these changes because of uncertainties in bioturbational effects^{14,15}, but we believe that the present estimates provide a reasonable working basis. The situation may be complicated by incomplete mixing, both laterally and vertically, and possible dominance of input from

FIG. 2 U_{37}^k temperature estimate from summer foraminiferal transfer function and $\delta^{18}O$ (*C. wuellerstorfi*)³ for terminations I, II and IV ($\delta^{18}O = \{(^{18}O/^{16}O)_{\text{sample}} / (^{18}O/^{16}O)_{\text{PDB}}\} - 1$). Termination I, interval 0 to 5 mbsf, composite depth, 0.5–25 kyr ago. Termination II, interval 13 to 21 mbsf, composite depth, 102–150 kyr ago. Termination IV, interval 46 to 51 mbsf, composite depth, ~318–343 kyr ago. a, Foraminiferal transfer function for summer (August). Average temperature of upper 150 m (down to below the thermocline). b, U_{37}^k , alkenone unsaturation index smoothed by a least-squares method²⁸; sea surface temperature, for top ~10 m of water (late spring). Termination I: between 1.6 and 2.2, and 3.4 and 4.6, the core was sampled every alternate 1 or 2 cm. The rest of the section shown was sampled every 8 cm. Between 13.5 and 20.2 mbsf (Termination II), and between 46.2 and 50.7 mbsf (Termination IV) the core was sampled every alternate 1 cm. c, Benthic $\delta^{18}O$ record of *C. wuellerstorfi*³. Numbers indicate isotopic stages³. Numbers in parentheses are 1,000 yr BP, based on



stratigraphic correlation of $\delta^{18}O$ events with several AMS ^{14}C -dated benthic curves³, corrected into calendar years^{26,29}, and with SPECMAP chronology¹.

intermittent algal bloom events. We recognise that more work is required to assess the statistical uncertainties of the SST estimates, because of possible small-scale heterogeneities in the sediment. Analysis of numerous replicate samples taken from single horizons down core should clarify this, as should examination of further cores in parallel.

Termination I. The most striking features of the SST curve (Fig. 2) for the Holocene and Termination I back to $\sim 21,000$ ^{14}C yr BP (equal to $\sim 24,000$ calendar years), as afforded by U_{37}^k measurements, are the intervals of rapid temperature oscillation and the overall lower temperatures as compared with earlier terminations. Over isotopic stage 2, the amplitude of the oscillations is twice those observed during the rise from the last glacial to the present interglacial (Termination I), both being sampled at the same rate of 1 sample every 2 cm. Unfortunately, earlier heavy sampling limited our sampling from 0.1 to 1.6 and from 2.2 to 3.5 m below sea floor across Termination I. Nevertheless, in the rest of the section, rapid fluctuations over intervals of 200–300 yr with amplitudes of more than 1.5°C are apparent, and at ~ 4.0 m below sea floor in stage 2, a fall of 3°C over 750 yr is followed by a rise of 5°C in $\sim 1,250$ yr. Estimates from the foraminiferal transfer function (summer) show some parallels, $\sim 1.5^\circ\text{C}$ higher than U_{37}^k .

Termination II. During late (benthic) $\delta^{18}\text{O}$ stage 6 and early stage 5, most SST- U_{37}^k events (Fig. 2) parallel the $\delta^{18}\text{O}$ climate stratigraphy, except for event 6.2 and, in part, the SST variability within event 5.4. In addition, the U_{37}^k signal slightly leads the

isotopic signal in several warming events, most noticeably around the boundary between isotopic stages 6 and 5. The transfer-function-based SST for summer overlies the U_{37}^k temperature record, but hardly parallels the molecular record or the $\delta^{18}\text{O}$ stratigraphy. These variations must be related both to the differing water depths at which the planktonic foraminifera and the coccolithophorid algae live, and to the unknown seasonal effect on the alkenone temperature signal. Figure 2 again illustrates one of our principal findings, which is that the surface water at site 658 has frequently warmed and cooled abruptly over short periods (for instance, up to 2.5°C over 300 yr). For comparison, the full range of glacial to interglacial SST- U_{37}^k change amounted to more than 6°C (from 19° to 25°C). In the upwelling region off Cape Blanc, such variations in SST most probably resulted from changes in the intensity of coastal upwelling, in turn the consequence of fluctuations in trade-wind strength²². Indeed, temperatures of 25°C are characteristic of the subtropical Atlantic outside the upwelling region²³. Peak warm stages, accordingly, correspond to a cessation of upwelling and trade winds. One of the cool-water events coincides with and thus corroborates the $\delta^{18}\text{O}$ cold event 5.52, a climate setback similar to that of the Younger Dryas³, during Termination II.

Termination IV. Once again, abrupt SST variations are apparent throughout the termination. There are possibly four steps of up to 3.0°C warming, and an abrupt temperature drop of 3°C after event 9.31, with the result that the overall warming before $\delta^{18}\text{O}$ event 9.3 appears to be slower than the subsequent cooling. The total SST shift estimated using the U_{37}^k data amounts to almost 6°C from glacial stage 10 to peak interglacial stage 9.3. As with Terminations I and II, little correlation is observed between the foraminiferal transfer-function temperatures and both the benthic $\delta^{18}\text{O}$ record and the SST- U_{37}^k curve. The difference between the SST- U_{37}^k and the transfer-function curves over the terminations may be due to a change in the dominant coccolithophorid species, for example where different species bloom at different seasons.

Numerous fast changes of up to 2.5°C over ~ 300 yr are apparent in all the terminations. Spectral analysis of the fast SST- U_{37}^k changes during Terminations II and IV (Fig. 3) indicates considerable periodicity, which accounts for almost 10% of the total variance, in the frequency range between 2 and 0.5 kyr^{-1} , far beyond that of the common Milankovitch cycles. Thus, it may indicate high-frequency forcing, hitherto little known²⁴, which is specific for each termination, with periods of ~ 600 yr and higher overtones of 1,150 or 1,280 and 1,610 or 1,800 yr at both Terminations II and IV. Minor power is also observed near 2.1, 3.3 and 4.4 kyr at Termination IV. Such oscillations may be linked to abrupt breakdowns in Atlantic deep-water ventilation resulting from meltwater events, as inferred from $\delta^{13}\text{C}$ data^{3,9,10}.

High-resolution U_{37}^k temperature studies promise to be especially useful in areas where the SST variations from glacial to interglacial times are believed to have been most pronounced, such as in key regions of the northwestern and northernmost Atlantic^{23,25}. If the still-controversial models of circulation and the salinity conveyor belt system^{3,9,10,26,27} are right, then one can predict that the movement of the ocean fronts will have left an extremely rapidly changing SST- U_{37}^k signal recorded in the bottom sediments. $\square$

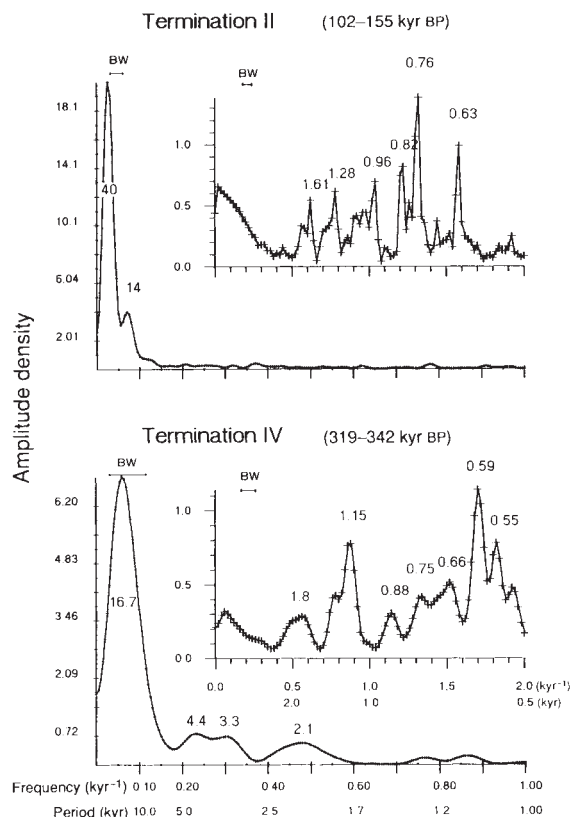


FIG. 3 Frequency spectra of U_{37}^k records at site ODP 658. a, Termination II, 102–155 kyr ago; b, Termination IV, 319–342 kyr ago. Spectra were derived using standard time-series procedures³⁰. Data were derived by gaussian interpolation (outer plots) and then treated with a triangular high-pass filter adapted from the SPECMAP project (insets). The method of spectral estimation used calculates the Fourier transform of the autocovariance function (Nyquist frequency of $1/500\text{ yr}^{-1}$)^{31,32}. Vertical axis is the power of spectrum. BW is the bandwidth. The high-pass-filtered spectra explain 97% of the variance at Termination II and 7.2% of the variance at Termination IV.

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A basalt trigger for the 1991 eruptions of Pinatubo volcano?

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THE eruptive products of calc-alkaline volcanos often show evidence for the mixing of basaltic and acid magmas before eruption (see, for example, refs 1, 2). These observations have led to the suggestion³ that the injection of basaltic magma into the base of a magma chamber (or the catastrophic overturn of a stably stratified chamber containing basaltic magma at its base) might trigger an eruption. Here we report evidence for the mixing of basaltic and dacitic magmas shortly before the paroxysmal eruptions of Pinatubo volcano on 15 June 1991. Andesitic scoriae erupted on 12 June contain minerals and glass with disequilibrium compositions, and are considerably more mafic than the dacitic pumices erupted on 15 June. Differences in crystal abundance and glass composition among the pumices may arise from pre-heating of the dacite magma by the underlying basaltic liquid before mixing. Degassing of this basaltic magma may also have contributed to the climatologically important sulphur dioxide emissions that accompanied the Pinatubo eruptions.

The 1991 eruptive activity at Pinatubo volcano (Luzon, Philippines) began with a series of steam and ash emissions which preceded (2 April–6 June) and accompanied the growth of a small lava dome (7–11 June)⁴. The first of four large pre-paroxysmal vertical eruptions took place on 12 June and produced tephra fall and small pyroclastic flow deposits. These vertical eruptions gave way to as many as 13 lateral blasts from multiple vents on 14–15 June. These, in turn, led to the paroxys-

mal eruption of 15 June, during which voluminous tephra fall and pyroclastic flow deposits were produced and a 2-km-diameter caldera formed.

A main component of tephra from the 12 June eruption is hornblende plagioclase scoria that contains accessory olivine, augite, quartz and anhydrite (Table 1). The bulk rock composition of this grey scoria is andesitic, with 59% SiO₂ (Table 2). Several lines of evidence suggest that the 12 June scoria represents a mixed magma. These include the presence of minerals that normally crystallize at widely differing temperatures (such as olivine and quartz); disequilibrium textures between crystals and glass; variable matrix glass compositions (ranging from 68 to 73% SiO₂ and averaging 70% SiO₂; Table 3); and the presence of magnesian olivine and clinopyroxene (Table 3). The olivine is too magnesian (Fo₈₇) to have been in equilibrium with the host andesite; Mg-Fe exchange equilibria^{5,6} indicate that the olivine crystallized from a basaltic magma with Mg/(Mg + Fe) = 0.68. Although we acknowledge the possibility that olivine xenocrysts were derived from the Zambales ophiolite beneath the volcano⁷, we favour a basalt source because of the relatively low nickel contents of the olivines and high titanium and iron contents of included spinel (Table 3).

We suggest that the 12 June scoria was produced by mixing of olivine basalt with crystal-rich dacitic magma. The silicic endmember was probably crystal-rich dacite that had been slowly cooling and crystallizing in a magma reservoir beneath the volcano since the last main eruptive period ~600 years ago⁸. The preservation of a disequilibrium mineral assemblage suggests that mixing took place shortly before eruption, although residence within the reservoir was of sufficient duration for the growth of coarse-grained reaction rims of hornblende on olivine (Fig. 1a).

Two types of juvenile dacite were erupted on 15 June: white crystal-rich pumice (47% crystals) and grey to tan pumice which is relatively poor in crystals (15–26%; Table 1). The two pumice types have similar bulk-rock compositions (Table 2) and similar phenocryst assemblages dominated by plagioclase and hornblende, the latter commonly rimmed by cumingtonite (Fig. 1b; Table 3), and both types contain anhydrite (ref. 9, Table 1).

TABLE 1 Modal data for 1991 pumice and scoria from Pinatubo volcano

Sample number	Σ (crystals)	pl	hb	cm	bt	ag	ol	ox	qz	ap	ahy	gm	ves
12 June 1991 andesite scoria													
7-1-91-1a	37.9	16.6	12.3	<0.5	<0.5	4.0	2.0	<1	<2	t	<2	62.1	53.4
15 June 1991 Crystal-rich pumice													
EW910615-1	47.0	31.3	12.1	<2	t			<2	<1	t	<0.5	53.0	60.8
15 June 1991 Crystal-poor pumice													
EW910615-2	26.3	20.3	5.5	<0.5			t	<0.5	<0.5	t	t	73.7	49.7
PH13-D	15.2	10.5	2.6	t				<1	<1	t	<0.5	84.8	58.9

Crystal and ground mass glass abundances recalculated on a vesicle-free basis. All values are in volume %. Abbreviations: ves, vesicles; gm, groundmass; pl, plagioclase; hb, hornblende; cm, cumingtonite; bt, biotite; ag, augite; ol, olivine; ox, oxides; qz, quartz; ahy, anhydrite; t, trace. Modes counted at magnification of ×400 on polished thin sections using combined reflected and transmitted light to distinguish vesicle walls.

TABLE 2 Major element analyses of pumice from Pinatubo volcano

Sample number	SiO ₂	Al ₂ O ₃	Fe ₂ O ₃	MgO	CaO	Na ₂ O	K ₂ O	TiO ₂	P ₂ O ₅	MnO	Total	SO ₃	LOI
12 June 1991 andesite scoria													
7-1-91-1a	59.2	16.1	6.13	4.86	7.17	3.91	1.56	0.64	0.28	0.12	100.00	0.95	0.28
15 June 1991 Crystal-rich pumice (white)													
EW910615-1	64.8	16.6	4.30	2.28	5.28	4.47	1.52	0.52	0.19	0.09	100.00		0.97
PIN1-3	64.2	15.6	4.43	2.49	5.20	4.64	1.56	0.48	0.16	0.11	100.00	0.42	0.77
15 June 1991 Crystal-poor pumice (tan)													
EW910615-2	64.3	16.6	4.37	2.53	5.47	4.42	1.47	0.50	0.18	0.09	100.00		1.01
PH-13-D	64.1	17.1	4.28	2.39	5.28	4.62	1.47	0.48	0.19	0.10	100.00	0.68	0.78

Major oxides by X-ray fluorescence analysis on fused lithium tetraborate disks and reported as weight per cent (volatile free). Sulphur determined on separate sample splits using a combustion-infrared technique. Results for sample PIN1-3 are averages for 3 analyses reported by Benard *et al.*⁹. All other samples analysed by D. F. Siems (for major elements) and J. Curry (for sulphur), both of the U.S. Geological Survey. 15 June samples are single pumice lumps, whereas the 12 June sample is a composite of several 1–3-cm scoria lapilli. LOI, loss on ignition.

The crystal-rich pumice typically has large (>1 mm) irregular and stretched vesicles, whereas the crystal-poor pumice has smaller (<1 mm) spherical vesicles and forms breadcrusted bombs. Inclusions of the crystal-rich pumice in crystal-poor pumice are relatively common, and the two types are mingled together in banded pumice blocks.

For the bulk-rock compositions to be similar (and non-eutectic), despite vastly different plagioclase and hornblende crystal contents, the matrix glass compositions in the two 15 June samples must be different. Microprobe analyses show that the matrix glass of the crystal-rich dacite is a highly evolved rhyolite with ~77% SiO₂, broadly similar to the most evolved matrix glasses in crystal-rich dacites from the Mount St Helens dome¹⁰. Matrix glass in the crystal-poor dacite is less evolved; the most crystal-poor sample examined has matrix glass with ~68% SiO₂, and comparatively high Al₂O₃, FeO, MgO and TiO₂ (Table 3). Crystals in the crystal-poor dacite show evidence of breakage, resorption and solution pitting (Fig. 1c, d).

We believe the crystal-poor dacite was produced mainly by heating of crystal-rich dacite and resultant crystal dissolution. Mass balance indicates that additional external components are not required. Dissolution of the dominant phenocrysts (hornblende and plagioclase) would drive the matrix melt back up the liquid line of descent to a composition lower in silica and less evolved, similar to the matrix glass in the crystal-poor-pumice. An alternative interpretation, that the crystal-poor dacite is simply a similar magma that has not yet crystallized is not viable because of the similar phenocryst assemblages, late-crystallized cumingtonite and similar mineral compositions in

both pumice types. It does seem strange that heating of the crystal-rich dacite to produce the crystal-poor variety did not result in the loss of one or more of the low-temperature phases. Possibly this reflects the short time between basalt intrusion and the 12 June eruption, and the different rates of mineral dissolution.

We propose the following model to explain the eruption of the andesitic 12 June scoria and the two pumice types of 15 June. Olivine-bearing basaltic magma intruded a shallow magma reservoir beneath the volcano. The magma reservoir contained crystal-rich dacitic magma, possibly left over from the previous eruptive episode ~600 years ago. The cumingtonite rims on hornblende indicate that the dacite was at ~800 °C (ref. 11). The relatively hot and dense basalt (~1,250 °C) ponded beneath the dacite and may have initially chilled at the interface¹². Heat transfer across the interface resulted in a thermal boundary layer in the dacite and may have promoted crystallization and exsolution of a fluid phase from the basalt. A temperature rise within the dacitic boundary layer caused crystals to dissolve and reduced the density and viscosity. Assimilation of the chilled interface and magma mixing of basalt and dacite produced volatile-rich olivine-bearing andesitic magma. Vapour saturation and vesiculation reduced the density of the mixed magma (see refs 1, 13), allowing ascent and eruption on 12 June. The paroxysmal eruption of 15 June followed, perhaps because the reservoir was depressurized by the initial mafic eruptions. In this sense, the intrusion of basalt and the ensuing mafic eruptions acted as a trigger for the paroxysmal eruption.

Our model requires a reduction of the solid fraction (crystals)

FIG. 1 a, Reaction rim composed of hornblende laths (hb) surrounding olivine (ol) in 12 June 1991 andesitic scoria; b, clear cumingtonite (cm) overgrowth on hornblende crystal in 15 June 1991 pumice; c, d, corroded margin of plagioclase grain (pl) in crystal-poor dacitic pumice erupted on 15 June 1991. Width of fields of view: (a, b) ~1 mm; (c) 0.34 mm; (d) 800 µm. a, b are transmitted light micrographs; c is a reflected light micrograph; d is a scanning electron micrograph.

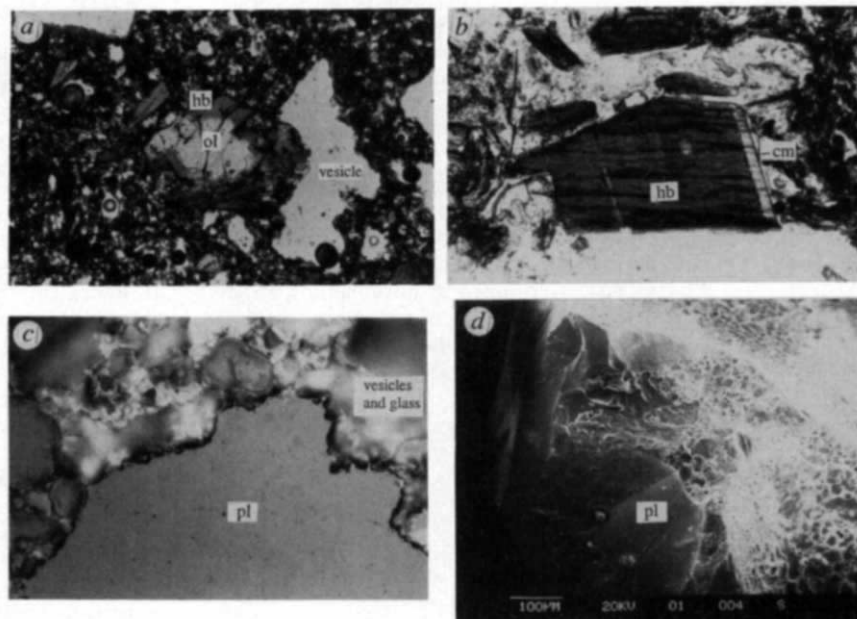


TABLE 3 Representative microprobe analyses of eruptive products

	SiO ₂	Al ₂ O ₃	FeO	MgO	CaO	Na ₂ O	K ₂ O	TiO ₂	MnO	Cr ₂ O ₃	NiO	Total	Mg#
7-11-91-1a 12 June 1991 andesite scoria													
ol	40.81	—	12.36	47.51	0.12	—	—	—	0.13	—	0.09	101.13	87.1
sp(ol)	0.25	16.22	33.75	10.88	—	—	—	1.11	0.11	35.52	—	97.83	52.1
ag	51.69	2.86	5.24	16.11	22.29	0.14	—	0.45	0.04	0.22	—	99.04	84.4
gl	mean (n=12)	70.39	14.96	2.41	0.72	2.57	3.18	0.31	—	—	—	97.37	
gl	s.t.d.	1.18	0.70	0.14	0.16	0.56	0.17	0.05	—	—	—	0.97	
EW910615-1 15 June 1991 Crystal-rich pumice (white)													
cm	54.59	1.64	16.72	21.30	1.77	0.16	—	0.12	0.79	—	—	97.11	68.4
hb	48.26	7.27	13.02	15.19	10.69	1.16	0.26	0.78	0.49	—	—	97.14	66.7
gl	mean (n=16)	76.55	12.47	0.75	0.10	1.20	3.52	0.06	—	—	—	97.72	
gl	s.t.d.	0.78	0.23	0.03	0.02	0.04	0.72	0.24	0.03	—	—	0.85	
EW910615-2 15 June 1991 Crystal-poor pumice (tan)													
cm	54.88	1.25	16.92	21.41	1.39	0.07	—	0.11	1.06	—	—	97.08	68.0
hb	48.13	7.39	13.12	15.48	10.24	1.24	0.20	0.93	0.43	—	—	97.12	67.1
gl	mean (n=6)	73.69	13.34	1.39	0.62	2.22	2.85	0.16	—	—	—	97.06	
gl	s.t.d.	3.27	1.26	0.87	0.72	1.50	0.55	0.48	0.13	—	—	1.08	
PH13-D 15 June 1991 Crystal-poor pumice (tan)													
gl	mean (n=5)	67.93	15.18	3.22	2.77	4.49	3.05	2.01	0.29	—	—	98.95	
gl	s.t.d.	0.79	0.54	0.25	0.71	0.52	0.87	0.17	0.04	—	—	1.42	

A dash indicates that the oxide was undetected or near detection limit ($\sim 0.05\%$). Abbreviations: ol, olivine; sp(ol), spinel in olivine; ag, augite; gl, glass; cm, cummingtonite. Analysed at 15 keV, 15 nA sample current (on brass). Spot size 20 μm on phenocrysts, point beam on microlites and glass bubble walls. Glass analyses corrected for migration of Na, K, Si and Al using iterative 1-s count data. $\text{Mg\#} = \text{Mg}/(\text{Mg} + \text{Fe} + \text{Mn}) \times 100$ (equivalent to forsterite content of olivine). Total iron reported as FeO, except for spinels, which are recalculated assuming $\text{R}^{2+}\text{R}_2^{3+}\text{O}_4$ formulae and the sum of FeO and Fe_2O_3 reported.

in the thermal boundary layer of 20–30 vol% to explain the difference in crystal contents of the crystal-rich and crystal-poor pumice types (Table 1). A minimum volume of 1 km^3 for the boundary layer is estimated from the proportion of crystal-poor dacite (20%) in the inferred erupted magma volume of $\sim 5 \text{ km}^3$ (ref. 14). Assuming a ratio of dacite melted to basalt crystallized of ~ 0.4 (ref. 15), a minimum of $\sim 1/2$ to $3/4 \text{ km}^3$ of basalt crystallization is required, so a much larger volume of basaltic magma must have been present to allow magma mixing. Involvement of a relatively large volume of basalt is also suggested by the large size of the magma chamber inferred from seismic data¹⁶. Preliminary analysis of the distribution of earthquake hypocentres that accompanied the Pinatubo eruptions suggests a magma body that extends from ~ 5 to $> 15 \text{ km}$ with a volume of $> 50 \text{ km}^3$.

The basaltic underpinnings of the magma reservoir may have contributed to the 20 megatons of SO_2 (ref. 17) emitted to the atmosphere during the Pinatubo eruptions. Given abundances of 500–1,000 p.p.m. sulphur in basaltic magmas¹⁸, degassing of a relatively large volume of basalt ($4\text{--}8 \text{ km}^3$) would be required for mass balance of the emissions. The origin of the atmospheric SO_2 is currently attributed either to degassing of an exsolved fluid phase in the dacite¹⁹, or to breakdown of anhydrite¹¹. We suggest that the ultimate source for the sulphur could lie at greater depth. The dacite may have been enriched in sulphur through upward migration of a fluid phase derived from basaltic magma at deeper levels of the volcano's feeder system.

Note added in proof: Andesite blocks derived from the 7–11 June 1991 lava dome contain abundant basaltic inclusions and provide strong support for the magma mixing model proposed here. A quenched inclusion from a sample provided by C. G. Newhall is magnesian ($\text{Mg\#} = 68$) dictytaxitic basalt with phenocrysts of olivine (Fo_{84-89} , rimmed by hornblende), augite and hornblende. Least-squares modelling of major-element data shows that a 40:60 mixture of this basalt and the crystal-rich dacite would produce mixed andesite that is virtually identical to the 12 June scoria. □

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The fungus *Armillaria bulbosa* is among the largest and oldest living organisms

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ASEXUALLY reproducing organisms occur in a variety of taxa in all biological kingdoms¹ and distinguishing asexually propagated genotypes is essential for the understanding of their population biology. Among the higher fungi, however, the clonal 'individual' is especially difficult to define² because most of the fungal thallus consists of a network of anastomosing hyphae embedded in the substratum. Whether fruit-bodies, the most recognizable part of a fungus, are produced by a single supporting mycelium can only be determined by establishing direct physiological continuity or genetic identity. We report a means by which individual fungi can be unambiguously identified within local populations and identify an individual of *Armillaria bulbosa* that occupies a minimum of 15 hectares, weighs in excess of 10,000 kg, and has remained genetically stable for more than 1,500 years.

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TABLE 1 Distribution of genetic markers in clone 1 monosporous isolates

	Monosporous isolate*															
	438-				445-				454-				455-			
	1	2	8	9	3	4	6	10	2	4	7	9	5	8	9	10
MATA	1	2	2	1	1	2	1	2	1	2	2	1	1	2	1	2
MATB	1	1	2	2	1	1	2	2	1	2	1	2	2	1	1	2
pVII-8	1	1	1	2	2	1	2	1	1	1	1	1	2	2	1	2
pVII-13	2	2	1	1	1	1	1	2	2	1	2	2	2	1	2	2
pVII-18	1	1	1	1	2	2	2	1	2	2	1	2	2	2	2	2
pVII-23	1	1	2	2	2	2	2	1	1	2	1	1	1	2	1	1
pJA1	1	2	2	2	1	2	2	1	2	2	1	1	1	1	1	1
R25-1	+	+	+	0	+	+	0	+	+	0	+	0	+	+	0	+
R25-2	0	+	+	0	+	0	0	+	0	+	0	+	0	+	+	+
R25-3	+	0	+	0	0	+	+	0	0	0	+	+	0	+	+	0
R25-4	+	0	+	+	+	+	+	+	0	0	+	+	+	+	+	+
R25-5	+	+	0	+	0	0	0	0	+	+	0	0	0	0	0	0
R25-6	0	+	+	+	0	+	+	0	+	+	0	0	0	0	0	0
R28-1	+	+	0	+	+	+	0	+	+	0	+	0	+	0	0	+
R28-2	0	+	+	0	0	+	0	0	0	+	0	0	0	0	0	0
R32-1	+	+	+	0	+	0	0	0	0	+	+	0	+	+	+	+
R32-2	0	+	+	+	0	+	+	0	+	+	0	0	+	+	+	+
R32-3	0	0	0	+	0	0	+	+	0	+	+	+	0	0	0	0

Each data set includes the four possible mating-types (MATA, MATB) segregating at meiosis from four fruit-bodies of clone 1. RFLP probes (pVII-8, -13, -18, -23) are anonymous nuclear DNA *EcoRI* fragments cloned in pUC18 from an *A. bulbosa* isolate by standard methods²⁷; pJA1 contains a portion of the nuclear rDNA repeat of *A. ostovae*²⁸. Of several RAPD primers tested, three gave multiple fragments: primer R25 (ACTTGAGGCG) was one of a series of arbitrary primer sequences that produced six, and primer R28 (ATGGATCCGC) produced two segregating fragments; primer R32 (CATCATCATCAT) is a repeated sequence found in a wide range of organisms (GenBank) and in the monosporous isolates examined produced 3 segregating fragments. RAPD fragments marking heterozygous loci segregated consistently in three independent experiments as present (+) or absent (0), and therefore act as dominant markers. To avoid bias, we chose marked loci that were not closely linked to one another, nor to either mating-type locus, which are always heterozygous. Allele designations are arbitrarily assigned for each RFLP, RAPD and mating-type locus.

* First number denotes fruit-body, second number denotes monospores isolate (see Fig. 1).

A. bulbosa is a facultative tree-root pathogen, commonly found in European and eastern North American mixed hardwood forests^{3,4}. It does not produce conidia or any other vegetative propagules but thalli form abundant macroscopic cord-like aggregations of hyphae, called rhizomorphs, which facilitate vegetative spread and food base acquisition in the forest floor.

Previous attempts to determine the size of individual clones of basidiomycetes have relied on two criteria: assay of somatic interactions⁵⁻⁷ and distribution of mating-type alleles^{8,9}. Somatic interactions suggest genetic dissimilarity when an 'antagonistic' reaction line develops along the contact zone between two isolates growing together, whereas isolates of the same or similar genetic constitution seem to mingle freely and establish a stable cytoplasmic continuum. The second method uses the bifactorial sexual incompatibility system found in most basidiomycetes including *A. bulbosa*¹⁰, in which compatible matings occur only when paired monosporous isolates have different alleles at two unlinked, multiallelic loci, designated *A* and *B* (ref. 11). It is then assumed that fruit-bodies with the same alleles at *A* and *B* loci and growing in 'reasonable' proximity are from the same diploid vegetative mycelium.

Using these criteria, it has been inferred that basidiomycete clones can exceed 500 m in diameter^{7,12,13}, which is surprising as mushrooms are perceived as spatially discontinuous and ephemeral. Unfortunately, neither method distinguishes a single large clone from a number of closely related individuals. As spore deposition decreases rapidly from the point of liberation¹⁴, mating between compatible spore offspring would generate a cluster of individuals that are genetically distinct but share the same four parental mating-type alleles. Furthermore, about half of such sib-related individuals may not be somatically antagonistic^{8,13}. To discriminate clearly between vegetative growth of an individual and multiple matings involving similar genotypes, we examined allelic composition at heterozygous loci marked by restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNAs (RAPDs) (ref. 15) in *A. bulbosa*.

In September of 1988 to 1991, *A. bulbosa* isolates (species

VII; ref. 3) were obtained from fruit-bodies and rhizomorphs along 1 km east/west, and north/south transects through a permanent study site in a northern Michigan hardwood forest. A region of 15 hectares yielded *A. bulbosa* isolates with identical mating-type alleles and mitochondrial DNA restriction fragment patterns, both of which are highly polymorphic within the species¹⁶. We provisionally refer to this group of collections as 'clone 1' (Fig. 1). If clone 1 is made up of inbred siblings, about half would show loss of heterozygosity at any unconstrained genetic locus that is heterozygous in the parent. In fact both allelic forms of each of five loci marked by RFLPs (Table 1) were clearly evident in Southern blot analyses of genomic DNAs from representative vegetative (diploid) isolates throughout clone 1. In addition, all 11 RAPD products, each marking a heterozygous locus (Table 1), were present in all vegetative isolates (Fig. 2).

The probability of retaining heterozygosity at each parental locus in an individual produced by a mating of sibling monosporous isolates is $P = (0.5)^m (0.75)^n$, where m is the number of co-dominant (RFLP) loci and n is the number of loci at which one allele is dominant (RAPD). In the case of clone 1, $P = (0.5)^5 (0.75)^{11} = 0.0013$. This low probability allows rejection of the hypothesis that the clone-1 isolates arose through independent matings. Indeed, the corresponding probability for either the 5 RFLP or the 11 RAPD markers taken separately, is <0.05 in each case. There is no obvious constraint toward heterozygosity at any of these loci unlike mating-type loci. Monosporous isolates showed normal ability to mate and produce homozygous mycelia in laboratory crosses. In addition, a neighbouring *A. bulbosa* clone (clone 2, Fig. 1) was homozygous at two of the RFLP loci, and lacked five of the clone 1 heterozygous RAPD fragments, implying homozygosity at these loci. Although the genetic markers used here are probably selectively neutral, it was not our goal to assess correlations of fitness and heterozygosity under natural conditions. The most acceptable remaining hypothesis is that clone 1 reached its present size through vegetative growth. As each clone occupies a discrete, contiguous territory and apparently excludes other clones of the same

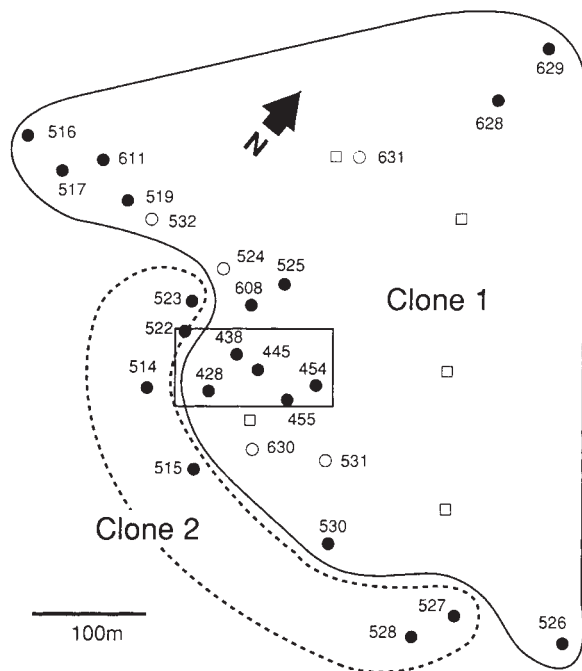


FIG. 1 Size and distribution of two *A. bulbosa* clones within a 30 hectare site. Cultures obtained from rhizomorph samples (608, 611, 628, 629) and subhyphenium of fruit-bodies (428, 454, 516, 517, 519, 525, 526, 530) were chosen for genetic analysis to represent vegetative, diploid isolates throughout 15 hectare 'clone 1' (solid line). Heterozygous loci (Table 1) were assayed in monosporous isolates from fruit-bodies 438, 445, 454 and 455. Additional *A. bulbosa* isolates (open circles) had clone-1 mating-type alleles and/or mitochondrial DNA type. No overlap of clone 1 and clone 2 (dashed line) was observed within an intensively sampled 0.8 hectare area (37 sample points within blocked area¹⁶). Linear growth rates of clone 1 rhizomorphs were determined by three independent methods: (1) rhizomorph growth along 45 poplar stakes buried within clone 1, measured after one growing season. The mean ($\pm$ s.d.) maximum length of the ten longest was 19.7 ± 3.1 cm; (2) maximum rhizomorph growth rates for clone 1 isolates on Weinhold's media with 0.24 g l^{-1} ethanol to enhance rhizomorph growth²⁵, at 8, 12, 15, 22 and 28 °C, were 0.0, 0.0, 2.1, 4.7, and 9.0 mm d⁻¹, respectively; (3) colonized 160 cm³ wood blocks with rhizomorph initials were placed in flats of topsoil taken from the study site and incubated at 10, 15 and 22 °C for 40 d. Respective mean maximum growth rates under these conditions were 0.2 ± 0.2 , 0.3 ± 0.3 , and 2.2 ± 0.4 mm d⁻¹ ($\pm$ s.d., $n=6$ for each temperature). Soil temperature at the study site approaches 14 °C at 10 cm depth and 14–16 °C at lesser depths for about 90 d per year. The annual growth rate of clone 1 is therefore estimated to be 20 cm by methods 1 and 2, and ≤ 6 cm by method 3. These are low compared to rhizomorph growth rates reported for *Armillaria* species^{6,29,30}, in keeping with a short growing season and heavy, prolonged snow pack in the area. To estimate the mass of clone 1 rhizomorphs 25 soil pits measuring (15 cm)³ were taken within clone 1 (open squares) and dissected to separate rhizomorphs from fine roots. The average dry weight of rhizomorphs from all soil pits was 0.1406 ± 0.1400 g per 225 cm², or about 6.25 g m⁻². This dry weight measure is equal to a fresh rhizomorph weight of 64.4 g m⁻² and mean rhizomorph length of 17 m per m² close to abundance estimates in other places²⁶.

species, dispersal of rhizomorphs, fruit-bodies or other mycelial fragments at the study site is unlikely. Other fungi that do produce vegetative propagules, exhibit an intermingling, mosaic pattern of genotypes in local areas^{17,18}.

Identifying 'clone 1' as a single genetic individual enables us to make minimal estimates of its age. Rhizomorph growth rates for clone 1 isolates were consistently ≤ 0.2 m per year (Fig. 1 legend). Estimating clone 1 to be 635 m across, its age is therefore about 1,500 years. This is probably an underestimate as antagonistic interactions would impede extension into areas occupied by other *A. bulbosa* clones. Clearly, clone 1 was established before the standing 60-year-old forest, and before a 300-year-old white pine/mixed hardwood forest that existed at the site before a 1928 fire. *Armillaria* rhizomorphs are known to withstand above-ground fires of extremely high temperatures¹⁹.

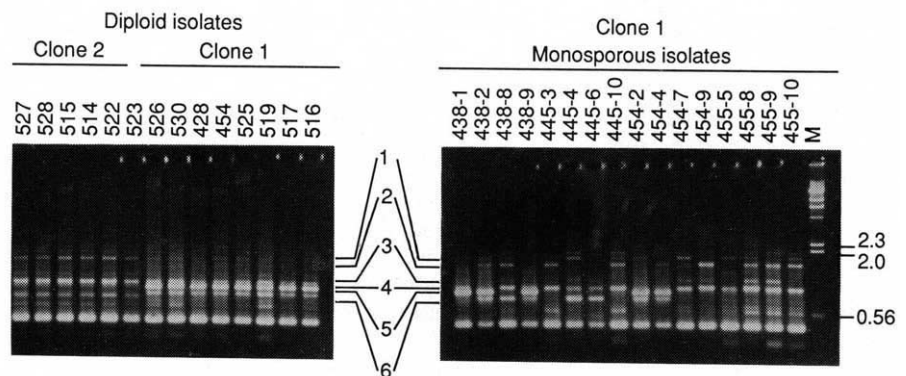
Given its age, the genetic stability of clone 1 is remarkable. In our study of heterozygous loci, a total of 20 RAPD and 27

nuclear DNA restriction fragments (including those in Table 1) were invariate in all diploid isolates of clone 1. This constitutes a direct measure of about 0.73 kilobases of nucleotide sequence (20 base pairs for each RAPD product; 12 base pairs for each *EcoRI* fragment assayed) and indicates the absence of DNA sequence rearrangements, insertions, deletions and other internal RFLP and RAPD site gains, which would be detected as polymorphisms. Our data also show no evidence for mitotic crossing over²⁰ with its attendant loss of heterozygosity, or for mutation.

Determination of reproductive success has not been made for any fungal individual because of the difficulties in tracking the dispersal and establishment of windborn spores²¹. As more than 10 *Armillaria* fruit-bodies can occur (on average) in a 5 m² area and a single basidiomycete fruit-body can distribute 1,000,000 spores per hour over several days²², the potential reproductive output of an individual the size and age of clone 1 is enormous. The successful colonization of areas already occupied by other

FIG. 2 RAPD products from primer R25 for monosporous isolates of clone 1 and diploid isolates of clones 1 and 2. Six fragments segregating for presence or absence in monosporous isolates are indicated in the centre, molecular size standards (lane M, in kb) are to the right.

METHODS. Polymerase chain reactions of 25 μ l volume included roughly 10 ng genomic DNA with 200 μ M each of dATP, dTTP, dGTP and dCTP, 1.0 μ M primer oligonucleotide (Regional DNA Synthesis Laboratory, Calgary, Alberta), and 2.5 μ l 10 $\times$ buffer with 0.75 units *Taq* DNA polymerase (Bio/Can, Ontario). Reactions used an initial 3-min denaturation at 93 °C, followed by 35 cycles of 37 °C for 1 min, annealing; 71 °C for 1 min, polymerization; 93 °C for 1 min, denaturation, in an M.J. Research FF100 Programmable Thermal Controller. Electrophoresis of 12- μ l post-reaction samples was in 1.5% agarose, 1 $\times$ TAE buffer²⁷ before



ethidium bromide staining and photography over a longwave ultraviolet light source.

clones of *A. bulbosa*, however, must be vanishingly rare. We observed rhizomorphs on 88 of 123 poplar stakes (72%) from a 130-m transect through clone 1 after one year, showing that new food bases are quickly colonized by the established clone.

Evidently, individuals of *A. bulbosa* are a substantial component of mixed oak forests. At the Michigan site, the fresh weight of clone 1 rhizomorphs in soil is estimated to be about 9,700 kg (Fig. 1 legend). This estimate does not include rhizomorphs below 15 cm, nor those associated with tree roots or other wood. Significantly, this value does not include the mass of fine hyphae in the soil and wood that necessarily supports rhizomorph growth. Depending on nutrient culture conditions^{23,24}, *Armillaria* hyphal mass may exceed rhizomorph mass 50-fold. In agar amended with hot-water extracts of forest soil, this ratio is 10.7 ± 4.4 ($\pm$ s.d., $n = 5$). In addition, *A. bulbosa* may occupy substantial volumes of suppressed or dead tree roots in oak forests²⁵, within which mycelia predominate. A conservative minimum mass estimate of the organism might therefore be 10 times that of the rhizomorphs, or, in the case of clone 1, about 100 tonnes, close to the mass of an adult blue whale. By comparison, *Sequoiadendron giganteum*, the largest plant on earth, attains a mass of over 1,000 tonnes, most of which is non-living wood that has accumulated during thousands of years of growth. In contrast to these more highly integrated organisms, fungi may undergo dramatic fluctuations in mass, for example, up to threefold within a few years, depending on recent forest history²⁶. This is the first report estimating the minimum size, mass and age of an unambiguously defined fungal individual. Although the number of observations for giant plants and animals is much greater, members of the fungal kingdom should now be recognized as among the oldest and largest organisms on earth. □

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Polymorphism in red photopigment underlies variation in colour matching

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GENETIC variation of human senses within the normal range probably exists but usually cannot be investigated in detail for lack of appropriate methods. The study of subtle perceptual differences in red–green colour vision is feasible since both photopigment genotypes and psychophysical phenotypes can be assessed by sophisticated techniques. Red–green colour vision in humans is mediated by two different visual pigments: red (long-wavelength sensitive) and green (middle-wavelength sensitive). The apoproteins of these highly homologous photopigments are encoded by genes on the X chromosome¹. Colour matches of males with normal colour vision fall into two main groups that appear to be transmitted by X-linked inheritance^{2–6}. This difference in colour matching is likely to reflect small variations in the absorption maxima of visual pigments^{7–11}, suggesting the presence of two common variants of the red and/or green visual pigments that differ in spectral positioning^{5,6}. We report that a common single amino-acid polymorphism (62% Ser, 38% Ala) at residue 180 of the X-linked red visual pigment explains the finding of two major groups in the distribution of colour matching among males with normal colour vision.

Fifty young Caucasian males with normal colour vision were recruited for this study. The determination of Rayleigh colour matches followed an algorithm¹² that measured the proportion of red (667 nm) in a mixture of red and green (551 nm) that was perceived as identical to a standard yellow (590 nm) light. The ranges of red/green mixtures accepted by subjects as matching the yellow comparison light, as well as the match midpoints (centres of match ranges) fell within the limits that define normal observers but were consistently different among observers. Some individuals clearly required more red whereas others required more green light to match the yellow comparison light.

The gross structure and coding sequences of the red and green pigment genes of each individual were determined by Southern blot^{1,13} and single-strand conformation polymorphism (SSCP) analyses followed by sequencing of polymerase chain reaction (PCR)-amplified DNA segments (Fig. 1). The results show that each subject had one red pigment gene and one or more green pigment genes as previously described^{1,13}. In addition, red and green opsins are both found to be highly polymorphic. A common polymorphism was observed at amino acid residue 180 of red opsin; 31 (62%) subjects had Ser (TCT), while 19 (38%) had Ala (GCT), indicating that two major forms of red opsins exist in the general population (Table 1), a possibility earlier surmised from microspectrophotometric measurements¹⁴. This Ser/Ala polymorphism was observed previously when the red opsin sequences of 3 individuals were determined¹. A frequency distribution histogram of the match midpoints for subjects with Ala or Ser at position 180 of red opsin is shown in Fig. 2. Higher sensitivity to red light (shown by a lower match midpoint in Fig. 2) is strongly correlated with the presence of Ser (Table 1).

Further evidence that the Ser/Ala polymorphism in red opsin is responsible for the variation in colour matching was obtained by studying nine deuteranopes who had no green pigment genes. Five of these individuals had Ser and four had Ala at position 180. The results were consistent with those in colour-normal

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observers in that the Rayleigh equation slopes fell into two groups that could be distinguished on the basis of the presence at position 180 of Ser (mean slope 0.063) or Ala (mean slope 0.0086; E.S. *et al.*, manuscript in preparation).

Two additional, but much less frequent, polymorphisms involving hydroxyl-bearing amino acids in the red opsin of colour-normal subjects were also observed. In one subject who had Ser and another who had Ala at position 180, Thr and Ser (typical of green opsin) instead of Ile and Ala were found at

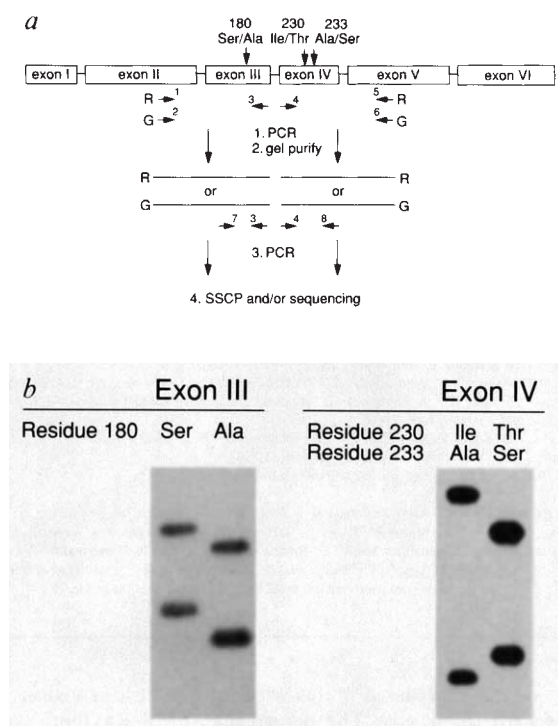


FIG. 1 Gene-specific sequence analysis of exons III and IV of the red and green opsin genes. *a*, PCR-amplification strategy. Arrows marked R or G represent red-specific or green-specific primers; unmarked arrows represent primers common to both red and green opsin genes. The number by each arrow refers to the primer sequence below. The indicated segments (lines) of the red and green opsin genes (boxes) were amplified (30-cycle PCR reaction of 1 min at 94 °C, 1 min at 64 °C and 3 min at 72 °C using the Perkin Elmer-Cetus Gene-Amp kit and DNA Thermal Cycler), and then purified by gel electrophoresis through a 1% low-melting point agarose (Seaplaque GTG FMC). The red- and green-specific segments were used as templates in a second PCR reaction (30 cycles of 1 min at 94 °C and 1 min at 64 °C) to amplify the downstream part of exon 3 and exon 4. The resulting DNA fragments were then analysed by SSCP¹⁹. The nucleotide differences responsible for altered mobility on SSCP were identified by dideoxy sequencing (Sequenase kit, US Biochemical) performed on PCR-generated single stranded DNA or on double stranded DNA after subcloning of amplified DNA into plasmid pGEM-3Z (Promega). Primers were synthesized on a PCR Mate model 380 DNA synthesizer (Applied Biosystems). The primer sequences shown in the diagram are: 1, 5'-CAGCATTGTGAACCAAGTCTC; 2, 5'-CAGCGTTGTGAACCAAGTCTAT; 3, 5'-CTGCTCCAACCAAGATGGGC; 4, 5'-TACTGGCCCCACGGCTGAAG; 5, 5'-GACGCAGTACGCAAGATC; 6, 5'-GAAGCAGAATGCCAGGACC; 7, 5'-TGCAAGCCCTTTGGCAATGTG; 8, 5'-CGCTCGGATGGCCAGCCACAC. Primers used to amplify the upstream part of exon 3 (not shown) are 5'-ATCACAGTCTCTGGTCTCTG and 5'-CACGATGGCCAGCTTGGCATC. Primers used for PCR amplification, SSCP analysis and sequencing of exon 2 are 5'-CACATCGCTCCAGATGGGT and 5'-ACACAGGGAGACGGTGTAGC and those for exon 5 are 5'-GTGGCAAAGCAGCAGAAAGAG and 5'-CTGCCGGTTCATAAAGACATAG. The position of non-homologous polymorphisms in the red pigment gene are indicated on top of the diagram. *b*, Autoradiograph of single stranded DNA fragments of exons III and IV of red pigment genes which have the indicated residues at positions 180, 230, and 233. The labelled single-stranded DNA fragments were separated on a 5% polyacrylamide gel (4.9% acrylamide/0.1% methylene-bis-acrylamide) containing 10% glycerol, 40W for approximately 5 h at room temperature (fan-cooled). Gels were dried on filter paper and exposed over night to X-ray film at -70 °C with an intensifying screen.

TABLE 1 Frequencies and average match midpoints for red pigment variants

Class	Residue at position			Frequency* (n=50)	Mean match midpoint (red/red + green)
	180	230	233		
I	Ser	Ile	Ala	0.60 (30)	0.455 ± 0.023
II	Ser	Thr	Ser	0.02 (1)	0.522
III	Ala	Ile	Ala	0.36 (18)	0.500 ± 0.018
IV	Ala	Thr	Ser	0.02 (1)	0.565

*Numbers of individuals are shown in parentheses.

The deduced amino acids at positions 180, 230 and 233 of red pigment apoproteins are shown together with the frequency and mean Rayleigh match midpoint ($\pm$ s.d.) of each of the classes observed. Methods are described in Fig. 2 legend. The match midpoints of classes I and III differ on average by 0.045 units with a 95% confidence interval of 0.057–0.033 units. A two-sample *t*-test (using the Minitab program, State College, Pennsylvania) showed that the mean match midpoints of classes I and III were highly unlikely to be identical ($P < 0.0001$; $t = -7.68$). Ser instead of Ala was present at position 180 of the green pigment in seven of the class I and one of the class III subjects. In addition, two of the seven class I subjects with Ser at position 180 of green opsin had Thr at position 65 instead of Ile. The match midpoints of these individuals were 0.404, 0.445 (class I, Thr) 0.415, 0.439, 0.445, 0.464, 0.470 (class I, Ile), and 0.499 (class III). The *P*-value obtained on comparing class I and class III subjects excluding those with Ser at position 180 in green opsin was also < 0.0001 ($t = -6.62$).

positions 230 and 233 respectively, generating two additional classes of red opsins as shown in Table 1. Figure 2 shows that, in comparison to others with the same amino acid (Ser or Ala) at position 180, these subjects required more red light in the mixture field to match the yellow standard. This finding is consistent with the presence of red pigments with absorption spectra shifted to shorter wavelengths.

Non-homologous amino-acid changes were also found at positions 65 and 180 in the green pigment opsins. At position 180, 42 (84%) of the colour-normal individuals had Ala, 4 (8%) had Ser and 4 (8%) had Ser in one but Ala in the other of their green opsins. Two (4%) individuals with Ser at position 180 of green opsin had Thr instead of Ile at position 65. Analysis of the Rayleigh match data set from which the eight individuals with Ser at position 180 were excluded suggests that substitutions in the green pigment are unlikely to contribute significantly to the observed variation in match midpoints (Table 1).

Polymorphisms that did not involve hydroxyl-bearing amino acids were found at positions 156 (Leu/Met), 171 (Val/Ile) and 236 (Met/Val) of red opsin and at positions 156 (Met/Leu), 174 (Ala/Val) and 178 (Ile/Val) of green opsin (J.W., Y. Hibuya, A.G.M. and S.S.D., manuscript in preparation). As determined by comparison of match midpoints, none of them accounted for the observed major phenotypic classes (data not shown).

Other factors that could lead to individual variation in the Rayleigh matches of our subjects are variations in lens density and in the optical density of the photopigments¹⁵. Because we tested a highly homogeneous sample of observers, variation in lens density is likely to be small. A reasonable assumption of variation in pigment optical density would also not account for the observed variation in Rayleigh matches⁶. The major differences in Rayleigh matches are therefore highly correlated with the Ser/Ala polymorphism at position 180 of red opsin. This X-linked polymorphism is expected to be subject to X-inactivation in females. Heterozygous females (47% of the female population) would therefore exhibit retinal patches with either Ser or Ala at position 180 of the red visual pigment. Consequently, these heterozygotes would show intermediate match midpoints as previously reported⁵.

The involvement of hydroxyl-bearing amino acids in spectral tuning of photopigments has recently been investigated in South American monkeys, including tamarins¹⁶. Unlike human males, these New World male primates have a single gene encoding a

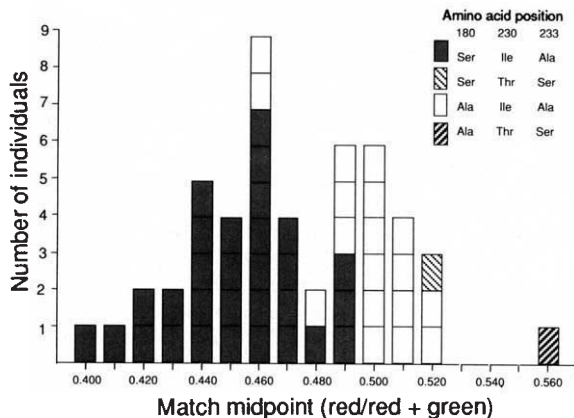


FIG. 2 Frequency distributions of the Rayleigh match midpoints and of the deduced amino acid sequence of the red pigment. Individual cases are shaded corresponding to the red pigment type expressed. Rayleigh match ranges were determined using a three channel maxwellian view apparatus similar to that described in Pokorny *et al.*²⁰ The stimuli appeared in a bipartite annular field with an eight degree outer and three degree inner diameter. The subjects observed the stimuli with their right eye while their head position was stabilized with a bite bar. The determination of the match was entirely under the control of a computer which presented the stimuli in an interleaved double staircase method as described¹². The ends of the match range were taken as the average of six reversals by each of the two staircases, after a criterion step size of 0.003 units was achieved. Unrecorded trials were randomly inserted to prevent subjects from detecting a pattern in the stimulus presentation. The stimuli were alternated with a uniform dark stimulus on a 1:1 sec duty cycle in order to maximize colour discrimination. The apparatus was calibrated after each experimental session using a photodiode. The test-retest correlation on a random subset of subjects ($n=10$) on this procedure was 0.950. Average match range width for the tested subjects was 0.0088 ± 0.0003 . The red and green values used to calculate red/red+green are the energies of the red and green primary, respectively.

photopigment with spectral sensitivity in the red-green range¹⁷. The results of these studies suggest that differences in absorption spectra among the red-green photopigments of these primates are determined by the additive effects of amino acid substitutions at three positions: Ser to Ala, Tyr to Phe and Thr to Ala at positions 180, 277 and 285, respectively. Furthermore, the tamarin pigments with absorption maxima at 556 nm and 562 nm differ only at position 180. The importance of the Ser/Ala polymorphism in specifying spectral characteristics of the human red pigment has been confirmed by direct determination of the absorption spectra of pigments expressed and reconstituted *in vitro*. The absorption maxima of red pigments with either Ala or Ser at position 180 were found to be at 552 nm and 557 nm, respectively¹⁸. These results support our finding that the Ser/Ala polymorphism at position 180 of red opsin explains the observed variation in red-green colour vision among people with normal colour vision. These findings provide a model to explain phenotypic differences in human sensory perception. □

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Absorption spectra of human cone pigments

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HUMAN colour vision is mediated by three light-sensitive pigments, each found in a different cone-cell type¹. The absorption spectra of the human cone pigments have been sought for over a century² using techniques such as psychophysical colour matching³, reflection densitometry⁴, electroretinography⁵, single-cell action spectra⁶ and, most directly, microspectrophotometry^{7,8}. We report here a direct determination of the human cone pigment photobleaching difference absorption spectra after the production of each cone pigment apoprotein in tissue culture cells transfected with the corresponding complementary DNA clones^{9,10}. The mean values for the wavelength of maximal absorption are 426 nm for the blue pigment, 530 nm for the green pigment, and 552 nm and 557 nm for two polymorphic variants of the red pigment.

Complementary DNAs encoding the blue, green and red human cone pigments as well as a red pigment variant identified in cloned genomic DNA⁹ (in which a serine replaces an alanine at position 180) were expressed in 293S cells. After *in vitro* reconstitution of the expressed apoproteins with 11-*cis* retinal and partial purification of the reconstituted pigment from unbound retinal, photobleaching difference absorption spectra of the cone pigments were obtained (Fig. 1a). In these difference spectra, the positive peak is derived from the photolabile visual pigment, whereas the negative peak at 380 nm arises from released all-*trans* retinal, a product of visual pigment photobleaching. For clarity, only one of the two red pigment difference spectra is shown in Fig. 1a. The long-wavelength limbs of both red pigment spectra are enlarged and overlaid in Fig. 1b.

The blue pigment absorption curve partially overlaps that of retinal, leading to two systematic distortions in the crude photobleaching difference spectrum. The first arises from residual 11-*cis* retinal in the membrane preparation, which is partly isomerized on illumination with the bleaching lights. To correct for this change in retinal absorbance, each blue pigment spectral determination was accompanied by a control photobleaching spectrum of an identically prepared membrane sample lacking visual pigment. The photobleaching difference spectrum of the control sample was subtracted from that of the experimental sample to give the spectrum shown in Fig. 1a. The second distortion results from the release of all-*trans* retinal by the bleached pigment. A corrected, more symmetrical blue pigment absorption curve can be produced by adding a scaled absorption curve of all-*trans* retinal to the difference spectrum (Fig. 1c).

For each difference absorption curve, the exact wavelength of maximal absorbance was determined by calculating the best fitting fifth-order polynomial to the 100-nm region of the spectrum centred about the approximate peak sensitivity. Mean wavelengths of maximal absorbance for multiple independent experiments are presented in Table 1. As a second method of comparing the red pigment spectra, the point of half maximal absorbance along the long-wavelength limb was also measured

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(Table 1). The mean absorption maxima for the blue, green and red Ala/Ser¹⁸⁰ (containing alanine or serine at position 180) cone pigments, 426 nm, 530 nm and 552/557 nm, respectively, may be compared with previously reported values obtained from the action spectra of single human cones (~ 530 nm and ~ 560 nm for green and red⁶), from electroretinography (530 nm and 561 nm for green and red⁵), from microspectrophotometry (419 ± 2 nm, 531 ± 3 nm and 558 ± 5 nm for blue, green and red⁸) and from psychophysics (435 nm, 534 nm and 560 nm for blue, green and red³).

The absorption maxima of the expressed red pigments with either Ala¹⁸⁰ or Ser¹⁸⁰ differ by 4.3 nm and the midpoints of their long-wavelength limbs differ by 4.8 nm (Fig. 1b and Table 1). These measurements are in good agreement with the prediction of 6 nm made from sequence comparisons of primate long-wavelength visual pigments⁵. The difference in absorption maxima between these two red pigments explains a number of observations. In a microspectrophotometric study of human red cones, the red peak sensitivities fell into two distinct groups, one with an absorption maximum of 554 ± 2.3 nm and the other, 563.2 ± 3.1 nm⁸. Four males tested fell into either one group or

the other. Psychophysical colour matching studies have found that Rayleigh matches made by males with normal colour vision also have a bimodal distribution^{11,12}, consistent with a polymorphic variation in red pigment absorption of several nanometres. In a recent study¹³ of 50 caucasian males with normal colour vision, the red pigment gene was observed to contain Ser¹⁸⁰ in 62% of the subjects and Ala¹⁸⁰ in 38%. This common polymorphism was found to be highly correlated with the bimodal distribution of Rayleigh matches in a manner consistent with the 4–5 nm spectral difference reported here. Finally, some dichromats who lack green sensitivity (deuteranopes) reject colour matches made by other deuteranopes, an observation that led Alpern and colleagues to hypothesize a family of red pigments, each member differing subtly from the others in its spectral position^{14–16}.

Expressed human cone pigments can now serve as a starting point for mutagenic studies to explore further the mechanisms of spectral tuning. They will also allow a precise definition of the spectral properties of variants that are likely to underlie differences in colour perception between individuals. Of particular interest are the spectral properties of the pigments encoded by hybrid genes formed by recombination events

FIG. 1 a, Superimposed difference absorption spectra of the human blue, green and red (Ala¹⁸⁰) cone pigments. One representative spectrum is shown for each pigment. b, Superimposed difference absorption spectra of the human red cone pigments containing either serine or alanine at position 180. c, A difference absorption spectrum of the blue cone pigment, uncorrected and corrected for released all-*trans* retinal. mOD, Optical density units $\times 10^{-3}$.

METHODS. The human cDNAs for the blue, green and red Ala¹⁸⁰ cone pigments (hs37, hs2 and hs7, respectively⁹) were inserted into the pCIS expression plasmid¹⁹. The red Ser¹⁸⁰ expression vector was created by site-directed mutagenesis of hs7 to replace Ala¹⁸⁰ with serine as described²⁰. The 5'-untranslated region of the red and green cone pigment cDNAs were replaced with the bovine rhodopsin 5' untranslated region –10 to –1 (ref. 21). The cone pigments were expressed in a human embryonic kidney cell line 293S (ATCC CRL 1573), after either transient transfection²⁰ or as stable lines created by cotransfection with a plasmid conferring neomycin resistance¹⁰. Stable lines expressing the cone pigments were identified first by RNA blot hybridization²² using a red or blue pigment cDNA probe, and then by reconstitution and spectral analysis of the expressed pigment. For each spectrum, stably expressing 293S cells grown in suspension (500 ml) or from transiently transfected 293S cells from 20 to 40 10-cm diameter dishes were processed as described previously²⁰ with the following modifications. In all steps, the buffer used was 50 mM HEPES, pH 6.5, 140 mM NaCl, 3 mM MgCl₂ and 2 mM EDTA (buffer A). The 250 mM sucrose solution contained additional protease inhibitors, 10 $\mu\text{g ml}^{-1}$ each aprotinin and leupeptin, and 1 mM DTT. Reconstitution of the cone pigments was accomplished by incubation of tissue culture cell membranes with a 20-fold molar excess of 11-*cis* retinal for 30 min to 2 h, before centrifuging in buffer A containing 4% BSA, which removes $\geq 95\%$ of the unbound 11-*cis* retinal. The membrane pellet was solubilized in 2% CHAPS in buffer A, and was either centrifuged at 10,000g for 5 min, at 86,000g for 10 min, or at 175,000g for 10 min before spectrophotometric measurements. Before photobleaching, four absorption spectra were measured and averaged. After photobleaching of the sample with a 150 W fibre optic light source (blue: 1 min through a 420-nm short wavelength cutoff filter; green: 2 min through a 580-nm narrow band pass filter; or red: 1 min through a 580-nm narrow band pass filter), four absorption spectra were measured and averaged, and the difference absorption spectrum was calculated by subtracting the averaged postbleach curve from the averaged prebleach curve. There was no evidence of absorption changes in the photoproducts during the 5-min period in which the four post-bleach absorption spectra were obtained. For each blue difference spectrum, a 293S cell pellet was processed in parallel, and the resulting 293S difference spectrum was subtracted from the blue difference spectrum (see text). Some difference curves contained a sloping background at shorter wavelengths due to an increase in light scattering as the spectra were collected. An empirically obtained background light scattering curve of 293S membranes was appropriately scaled and subtracted from those curves requiring background correction to equalize the absorbance values at 300 and 700 nm. An absorption curve of all-*trans* retinal in 2% CHAPS, buffer A, was scaled and added to each blue difference curve to eliminate the effect of the released all-*trans* retinal.

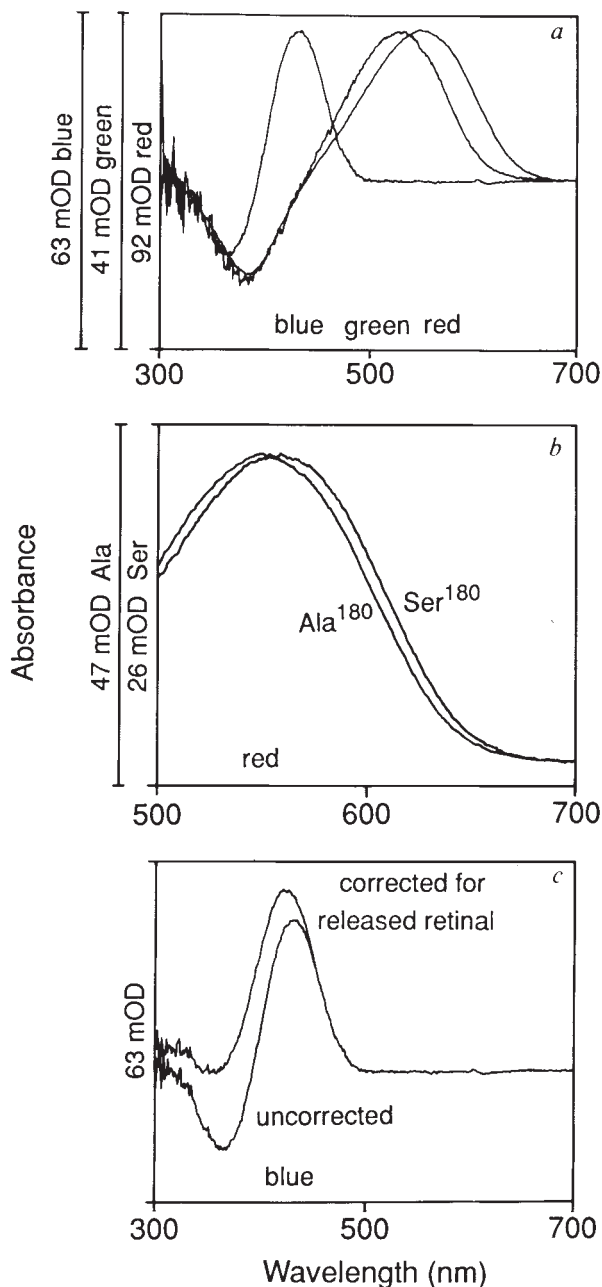


TABLE 1 Quantitative analysis of cone pigment spectra

	Mean λ_{max} (nm)	s.e.m. (nm)	s.d. (nm)	Number of experiments (n)	Mean amplitude (mOD)		Mean λ_{2max} (nm)	s.e.m. (nm)	s.d. (nm)
Blue	432.5	0.6	1.4	6	17.3	Red Ala ¹⁸⁰	607.3	0.3	0.8
Blue corrected for released retinal	426.3	0.4	1.0	6	21.3	Red Ser ¹⁸⁰	612.1	0.3	0.7
Green	529.7	0.8	2.0	6	12.0				
Red Ala ¹⁸⁰	552.4	0.4	1.1	7	39.3				
Red Ser ¹⁸⁰	556.7	0.8	2.1	7	15.0				

Absorption difference spectra were obtained as described in Fig. 1. For each difference absorption curve, the exact wavelength of maximal absorbance (λ_{max}) was determined by calculating the best fitting fifth-order polynomial to the 100-nm region of the spectrum centred about the approximate peak sensitivity. The wavelength of half-maximum absorbance (λ_{2max}) along the long-wavelength limb of the 14 red pigment spectra was determined from the best fitting 5th-order polynomial for each absorption curve between 500–650 nm. Curve fitting was done using a Macintosh computer and Cricket Graph software. The mean correlation coefficient (r^2) for the fifth-order polynomials was 0.992.

s.e.m., standard error of the mean.

s.d., standard deviation.

mOD, Optical density units $\times 10^{-3}$.

between red and green pigment genes. The hybrids are found in 6–8% of caucasian males who have anomalous trichromacy and are predicted to encode pigments with anomalous absorption properties¹⁷.

Following submission of this manuscript, Oprian *et al.*¹⁸ have also reported a similar analysis of recombinant human cone pigments. □

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Normal dystrophin transcripts detected in Duchenne muscular dystrophy patients after myoblast transplantation

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GENE delivery by transplantation of normal myoblasts has been proposed as a treatment of the primary defect, lack of the muscle protein dystrophin, that causes Duchenne muscular dystrophy (DMD), a lethal human muscle degenerative disorder^{1,2}. To test this possibility, we transplanted normal myoblasts from a father or an unaffected sibling into the muscle of eight boys with DMD, and assessed their production of dystrophin. Three patients with deletions in the dystrophin gene expressed normal dystrophin transcripts in muscle biopsy specimens taken from the transplant site one month after myoblast injection. Using the polymerase chain reaction we established that the dystrophin in these biopsies derived from donor myoblast DNA. These results show that trans-

planted myoblasts persist and produce dystrophin in muscle fibres of DMD patients.

Duchenne muscular dystrophy is an X chromosome-linked muscle disorder caused by mutations in the dystrophin gene (which spans 2,000 kilobases)^{3–5}. In about 65% of patients the mutation is a deletion or duplication which can be detected using the polymerase chain reaction (PCR)^{6,7}. The remainder are presumably point mutations. Dystrophin, the gene product⁸, is absent from the muscle of DMD patients⁹ except in cases of reversion, where second site mutations lead to the expression of truncated proteins^{10,11}. A therapeutic strategy for delivering dystrophin to muscles of DMD patients by myoblast transplantation was suggested by experiments in rodents. These experiments showed that myoblasts could cross a thick meshwork of extracellular matrix components, previously thought to be impermeable. In the course of normal development, myoblasts traverse the basal lamina and fuse randomly with all fibres in their vicinity^{12,13}. Myoblasts grown in culture and injected into muscle also gain access to and become an integral part of mature muscle fibres of the host, both in normal^{14,15} and in dystrophic animals^{1,16}. In the mdx mouse, the animal model for DMD, myoblast transfer and direct DNA injection have led to dystrophin production^{1,17}. Here we show that in patients with DMD, injection of human myoblasts leads to normal dystrophin production.

All experiments were conducted double blind: neither the members of the team involved in analysing the results, nor the patients and their families were aware of which leg received cells and which received placebo. Pre-implant muscle biopsies taken from eight DMD patients confirmed the presence of deletions in the dystrophin gene previously identified in lymphocytes using the multiplex PCR assay¹⁸. For transplantation, myoblasts were isolated from the muscle biopsy of a first degree

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relative, purified to 95% by fluorescence activated cell sorting and grown from 10^4 to 10^8 cells as described^{2,19,20}. Myoblasts were injected into patients at 80–100 sites in the tibialis anterior of one leg; placebo was similarly injected into the contralateral leg (Table 1).

One month after transplantation, two muscle biopsies, one superficial and one deep within the muscle, were obtained from each leg of each patient and analysed for the presence of dystrophin messenger RNA after reverse transcription and PCR. Two sets of primers were used for amplification that were specific for the N-terminal or C-terminal regions of the dystrophin molecule (Fig. 1). For each patient, pre-transplant muscle biopsies served as a negative control. Amplification of undelated regions of the gene served as a positive control for PCR reactions and complementary DNA integrity, because the dystrophin gene

is still transcribed even if it is not translated into a functional protein.

Biopsies from patients 1–6 with deletions in a C-terminal region of dystrophin were analysed by PCR using primers 7A/48R (Fig. 1a). In all four biopsies of the myoblast-injected (M) leg of patients 5 and 6, donor transcripts were detected (Fig. 1a, left). No transcripts were detected in the four biopsies of the placebo-injected (P) legs. Southern blot analysis of the amplified products confirmed that the bands contained the sequence recognized by probe 1 (Fig. 1a, right). As primer set 7A/48R spanned several exons (43 to 48), these results indicated that one month after injection into patients 5 and 6, donor myoblasts were alive and transcribing normal dystrophin.

Critical to the PCR analysis was the selection of primers. When one primer (in this case 48R) was within the deleted exon,

FIG. 1 Detection of dystrophin production by PCR analysis in three patients one month after myoblast transplant. For each leg of each patient, the results are representative of two different biopsies, one superficial and one deep within the muscle. **a**, Patients 5 and 6 (top) had a deletion in exon 48. Primer set 7A/48R amplifies a 842-bp product (primer 48R 5'-AGGAGATAAC-CACAGCAGCAG-3', primer 7A (ref. 22)). Oligonucleotide probe 1 (5'-AGACCTTGGGCAGCTTGA-3') recognizes a sequence (exon 48) in the PCR product amplified by 7A/48R. Ethidium bromide staining (bottom left) of PCR products amplified with primers 7A/48R, and Southern blot using probe 1 (bottom right). A band of normal molecular weight recognized by probe 1 is detected in the muscle of the myoblast-injected leg (M) but not the placebo-injected leg (P) from both patients. Because it spans several exons, this band was derived from mRNA. Leftmost lane, *Hae*III digest of Φ X174 DNA size markers (bp); Pre, pre-transplant biopsy control; C, normal control. **b**, Patient 8 had a deletion in exons 8–19 and patient 7 had a deletion in exons 8–12 (top). Primer set 2A/2B (ref. 22) amplifies a 1,329-bp fragment in the N-terminal region of dystrophin. Oligonucleotide probe 2 (5'-ATGGAG-GAAGAGCCTCTT-3') recognizes a sequence within the PCR product amplified by 2A/2B (exon 12). In the muscle samples of patient 8 but not patient 7, PCR amplified a normal sized product from the myoblast-injected leg (M) containing the sequence recognized by probe 2: ethidium bromide staining (left) of PCR products amplified with primers 2A/2B and Southern blot using probe 2 (right). Lane designation as in **a**. Primers and probes were synthesized and purified by HPLC (Operon Technologies). Probes were labelled at their 5' end with a biotin-avidin-horseradish peroxidase complex (Operon). **METHODS.** Muscle biopsies were quickly washed in cold sterile PBS, frozen in liquid nitrogen and stored at -70°C . Frozen tissue was pulverized with a mortar and pestle in the presence of liquid nitrogen and the total RNA extracted following the 'RNAzol' method (Cinna Biotecx)²⁹. For reverse transcription-PCR, cDNA was obtained by reverse transcription of 0.1 μg total RNA in a 10- μl reaction as described³⁰. For a 50- μl PCR reaction, 10 μl cDNA mix was combined with 4 μl 10 $\times$ PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl₂ 0.01% (wt/vol) gelatin), (Perkin-Elmer) 1.25 U *Taq* polymerase (Ampli Taq ; Perkin-Elmer) and 0.1 μM of each set of primers. The PCR protocol was: heated at 93°C for 60 s, 60°C for 60 s and 72°C for 2 min for 40 cycles in a DNA Thermo Cycler (Perkin-Elmer). Five microlitres of each amplified product was run on a 1.5% Nusieve (FMC Bio-Products), 0.5% agarose gel, Southern-blotted and hybridized as described³⁰, with 0.25 pmol ml⁻¹ of non-radioactive oligonucleotide probe 1 or 2 for one hour at 42°C . After hybridization, membranes were washed with 1 $\times$ SSPE (0.02 M EDTA, 0.2 M NaOH, 0.2 M NaH₂PO₄, 3.6 M NaCl, pH 7.5), 1% SDS for 30 min, 0.1 $\times$ SSPE, 1% SDS for 20 min and PBS for 5 min at room temperature. Each filter was then developed using the ECL Gene Detection System (Amersham, Illinois).

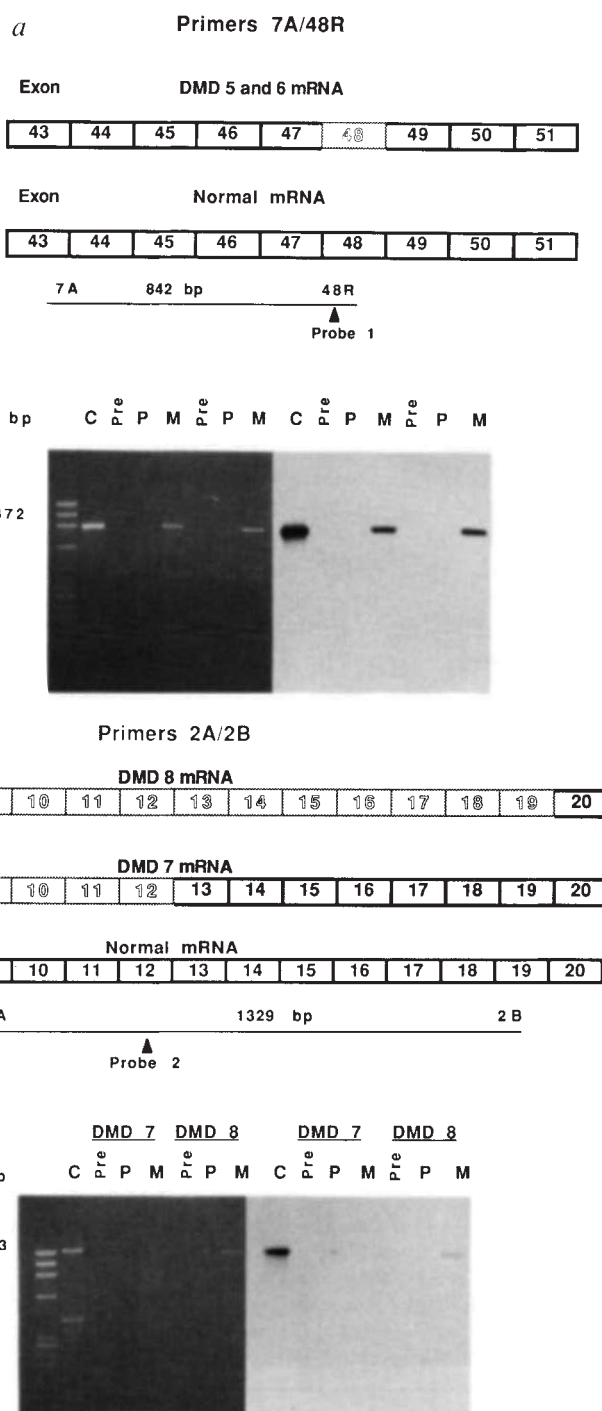


TABLE 1 Exons detected one month after myoblast transfer

Patient	Patient age	Donor age		HLA†	Number of dystrophin-positive fibres post-implant‡		Exons missing pre-implant§	Exons detected post-implant§		Exons detected post-implant§	
					Myoblast	Placebo		Myoblast	Placebo		
5	6	43	Donor	Class I	A11 A24 B7 B27 Bw4 Bw6 Cw2 Cw7	11	0	48	48	—	
				Class II	DRw15 DQw1						
			Patient	Class I	A2 A24 B7 B27 Bw4 Bw6 Cw2						
				Class II	DRw15 DQw1						
6	8	31	Donor	Class I	A2 B7 Bw6 Bw60	6	0	48	48	—	
				Class II	DRw6 DRw15 DRw52 DQw1						
			Patient	Class I	A2 A9 B39 Bw6 Bw60						
				Class II	DR4 DRw6 DRw52 DRw53 DQw1 DQw7						
8	7	2	Donor	Class I	A2 B7 B44	1	1	8–19	9–19	—	
				Class II	DR2 DR4						
			Patient	Class I	A2 B7 B44						
				Class II	DR2 DR4						
1	10	42	Donor	Class I	A2 B13 B44 Bw4	4	10	48–52	—	—	
				Class II	DR7 DRw11 DRw52 DRw53 DQw2 DQw7						
			Patient	Class I	A2 B13 B44 Bw4						
				Class II	DR7 DRw11 DRw52 DRw53 DQw2 DQw7						
2	10	42	Donor	Class I	A11 A24 B15 B18 Bw4 Bw6	11	12	45–52	—	—	
				Class II	DR2 DQw1						
			Patient	Class I	A11 A24 B15 B18 Bw4 Bw6						
				Class II	DR2 DR3 DRw52 DQw1 DQw2						
3	9	9	Donor	Class I	A24 A29 B7 B35 Cw7 Bw6	3	7	48	—	—	
				Class II	DR2 DR4 DRw53 DQw1 DQw7						
			Patient	Class I	A24 A29 B7 B35 Cw7 Bw6						
				Class II	DR2 DR4 DRw53 DQw1 DQw7						
4	7	34	Donor	Class I	A3 A29 B7 B44 Bw4 Bw6	1	2	48	—	—	
				Class II	DR7 DRw15 DRw53 DQw2 DQw6						
			Patient	Class I	A2 A29 B44 Bw4 Bw58 Cw6						
				Class II	DR4 DR7 DRw53 DQw2 DQw7						
7	6	29	Donor	Class I	A24 A29 B44 Bw4 Bw6 Bw62 Cw9	ND	ND	8–12	—	—	
				Class II	DR7 DRw11 DRw52 DRw53 DQw2 DQw7						
			Patient	Class I	A24 A30 B14 Bw6 Bw62 Cw8 Cw9						
				Class II	DR1 DRw11 DRw52 DQw1 DQw7						

* Donors were screened for the absence of infectious disease and for HLA match. Muscle tissue (0.5–1.0 g) was removed under local anaesthesia from the biceps of donors. Myoblasts were isolated from dissociated tissue, purified using the fluorescence activated cell sorter, grown to 10^8 cells^{2,19,20} in a quality-controlled commercial facility (Somatix), and tested for microbial contamination before use in patients. 10^8 purified myoblasts were injected into the tibialis anterior muscle of each patient. Between 80–100 injections were delivered in a grid (4 × 6 cm) spaced 5 mm apart. At each site myoblasts were delivered continuously along a track that spanned 2.5 cm in depth. Contralateral legs received a similar number of injections with placebo (saline and human serum albumin only). Post implant biopsies (1 cm³) were from a single track that received at most 5×10^6 myoblasts.

† HLA class I and class II phenotypes are listed for each donor/patient pair. Patients 1, 2, 3 and 8 have a perfect HLA match with the respective donor. Mismatched genes in donors which could be recognized by the hosts are indicated in bold typeface. All patients were immunosuppressed (oral dose of cyclosporine was 5 mg per kg per day).

‡ For immunofluorescence analysis, an average of 20 serial sections of ~1,000 fibres each were analysed per biopsy using two different antibodies (Fig. 2). Values are the maximum number of positive fibres detected in a single section of each biopsy.

§ Deletions in the dystrophin gene were determined by PCR on genomic DNA from lymphocytes¹⁸ and were confirmed by RNA analysis of pre-implant muscle biopsies. Bold-face numbers refer to the exons missing in the pre-implant and placebo-injected muscle biopsy samples, which were transcribed one month after myoblast transplantation. For C-terminal deletions, primers to the N terminus served as positive controls for the PCR reaction and cDNA integrity; for N-terminal deletions, primers to the C terminus served as positive controls.

only full-length donor derived mRNA was amplified, as shown here (Fig. 1). However, when the primers flanked the deleted exon(s), only truncated patient mRNA was detected, even in the presence of donor mRNA (data not shown). This was presumably due to preferential amplification of the more abundant shorter transcript from the patient (E.G. *et al.*, manuscript in preparation).

The missing exons were not detected in the mRNA derived from the biopsies of patients 1–4 (Table 1). Thus, normal dystrophin transcripts were either absent or present at less than 1.6 fg dystrophin mRNA/microlitre of total RNA, the approximate limit of detection of the assay as determined by progressive dilution, given the reported abundance of dystrophin transcripts²¹ (data not shown).

A similar analysis was performed for patients 7 and 8, who had deletions in the N-terminal portion of dystrophin (Fig. 1b). cDNA amplification of mRNA from muscle biopsies using primers 2A/2B (ref. 22) which span multiple exons of dystrophin, revealed a band corresponding to a size similar to that of the normal control in two biopsies of the myoblast-injected leg of patient 8. By contrast, no transcripts were detected in biopsies from the placebo-injected leg of patient 8 or either leg of patient 7 one month after myoblast transplantation. Thus, myoblast transfer led to dystrophin production in patient 8, but not in patient 7.

Biopsies were also analysed for dystrophin by immunohistochemistry. On average, 20 serial transverse sections of 1,000

fibres were analysed per biopsy. For patients 5 and 6, the dystrophin positive fibres were observed only in the leg injected with myoblasts, but not in the leg injected with placebo, and the dystrophin immunoreactivity was localized to the sarcolemma of mature fibres (Fig. 2). At the site of injection, between one and eleven positive fibres were detected. The frequency of positive fibres in the plane of a single section was ~1%, similar to that observed after direct injection of DNA into muscle of dystrophic mice¹⁷.

Although the immunofluorescence results correlate well with the PCR results in patients 5 and 6, the immunofluorescence assay does not provide definitive evidence of successful myoblast transfer. For example in patients 1–4, PCR failed to detect dystrophin transcripts that included the missing exons, and comparable numbers of immunoreactive fibres were detected in both placebo- and myoblast-injected legs (Table 1). Possibly, dystrophin was detected by immunofluorescence because of reversion events, second site mutations that caused in-frame transcripts to be produced which lacked the deleted exons. Currently, full-length dystrophin protein cannot be distinguished from truncated protein. That would require antibodies which recognize peptides in their native conformation encoded specifically by donor exons that are deleted in patients, reagents that are not yet available. By contrast, PCR analysis of mRNA clearly shows that the gene product contains the missing exons. It is unlikely that a secondary mutation could lead to the spontaneous replacement of a large portion of deleted DNA,

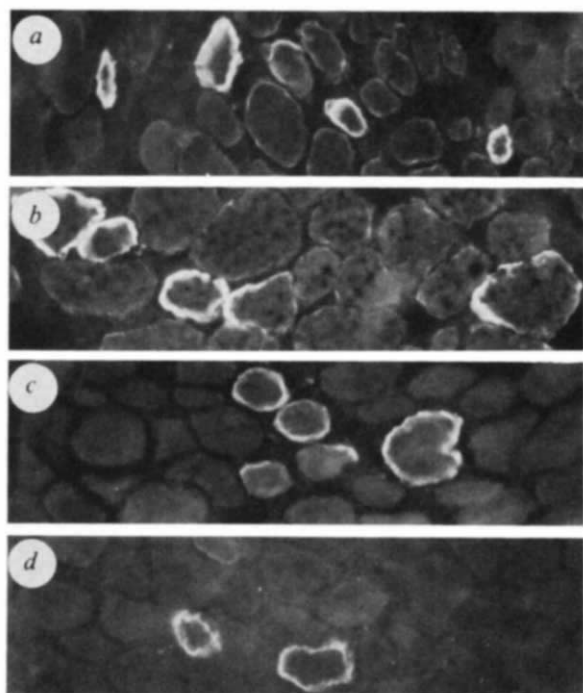


FIG. 2 Detection of dystrophin positive fibres in biopsy sections one month after transplant. Positive fibres are shown for biopsies from myoblast-injected legs of patient 5 (a, b) and patient 6 (c, d). As many as 11 positive fibres with dystrophin domains up to 0.5 mm in length were detected at these sites by serial sectioning. No positive fibres were observed in the contralateral placebo-injected legs (Table 1).

METHODS. Frozen 10- μ m serial sections were collected on gelatin-coated slides and stained with supernatants of the monoclonal anti-dystrophin antibodies, Man DYS1 (ref. 31) and ManDRA1, which recognize the rod and carboxyl terminus, respectively. Between 24 and 48 sections were analysed with each antibody. Sections were incubated with antibodies for 1 h, washed 3 times with PBS and 0.2% Tween-20 for 5 min each, blocked with PBS and 10% horse serum for 1 h, incubated with a biotinylated antimouse IgG (Vector Labs; 1/200 dilution), washed as described, and then incubated with Texas red-streptavidin (Zymed; 1/1500 dilution). All incubations were at room temperature. After a final wash step, slides were mounted in Gelvatol and analysed with a Zeiss Axiophot microscope with epifluorescence.

for example 12 exons in patient 8. These exons are therefore presumably derived from injected donor myoblast DNA. One limitation of PCR, however, is that it can still not be readily applied when the primary defect in the DMD gene is a point mutation, which occurs in 35% of cases.

The goal of myoblast transfer as a therapy for DMD patients is to produce heterokaryon fibres that are mosaics of nuclei, some of which do and some of which do not express dystrophin^{1,2,23,24}. Such fibres occur naturally in obligate carriers of DMD²⁵⁻²⁸ owing to lyonization of the X chromosome early in development and, with few exceptions, these individuals are free of symptoms. Our results indicate that dystrophin transcripts specific to the normal donor can be produced in the muscles of DMD patients one month after myoblast injection. These findings are encouraging, but the efficiency of myoblast transfer remains low. The HLA match of donor/patient pairs did not seem to influence the results in the presence of cyclosporine (Table 1). Age may be a factor: the three patients with detectable dystrophin were among the youngest with the least muscle fibrosis. Cell loss is also likely: the proportion of transplanted cells retained, their distribution, and their access to fibres have not been determined in humans. Genetic marking may be essential to follow the fate of transplanted human myoblasts more precisely^{14,15} and optimize delivery. If the efficiency is increased, myoblast transplantation should prove useful not only for the delivery of muscle proteins, but also for systemic delivery of recombinant non-muscle proteins by genetically engineered

myoblasts^{14,15} in the treatment of a number of acquired and inherited human diseases.

Note added in proof: For patient 5 we now have evidence by PCR of mRNA produced by donor myoblasts 6 months post-transplant. The muscles of the other patients have yet to be assayed at this time point. □

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Surface particle transport mechanism independent of myosin II in *Dictyostelium*

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CELLULAR locomotion could be driven by the rearward transport of membrane-bound particles observed on motile fibroblasts¹, keratinocytes² and neuronal growth cones³. A force propelling free surface particles backwards could move the cell forwards if the particles were anchored to a rigid substratum. During capping, myosin II ('double-headed' myosin) draws crosslinked membrane proteins to the rear of a cell. The *mhcA*⁻ mutant of the amoebal stage of the slime mould *Dictyostelium discoideum*, in which the myosin II gene has been deleted⁴, cannot cap surface particles but can crawl along the substratum^{5,6}. Thus, the mechanism driving capping is not essential for locomotion. We show here that the null mutant is capable of a different type of active rearward transport, independent of myosin II and distinct from capping. The transported particles on *mhcA*⁻ cells follow parallel paths. In the wild-type Ax2 strain, myosin II causes particles to converge towards a focal point and significantly increases the velocity of transport behind the leading edge of the cell.

Transport of surface particles on *Dictyostelium* amoebae has been described previously^{7,8}. Figure 1 shows our observations

on Ax2 cells using concanavalin A (conA)-coated beads. Initially, the beads are distributed randomly (Fig. 1a), but after several minutes a compact cluster of beads forms on many cells (Fig. 1b). Although *mhcA*⁻ cells cannot cap soluble conA, they do transport beads (Fig. 1c, d). In contrast to the wild type, beads are typically aligned in a row on the cell's rear edge by a process independent of myosin II (Fig. 1d). In both Ax2 and *mhcA*⁻ cells, 10 μ M cytochalasin A inhibits bead transport, suggesting a requirement for an intact actin cytoskeleton. Cytochalasin A also causes cells to become rounder, inhibits capping in Ax2 cells and disrupts the actin cytoskeleton (determined by rhodamine-phalloidin staining; data not shown).

Time-lapse recordings of individual cells reveal that beads are transported in the opposite direction to cell translocation or pseudopod extension (Fig. 2). Of 120 Ax2 cells examined, 118 demonstrated rearward transport; the other two cells were non-motile with beads capping on one cell but not on the other. Rearward transport was observed in 99 of 117 *mhcA*⁻ cells. Of the 18 cells that did not transport beads, nine had no motile activity. The remainder were either non-polarized cells with pseudopods distributed around the cell or bipolar cells with pseudopods at both ends; in each case beads were centrally located, as if driven by opposing forces. The occurrence of bead transport on stationary cells with pseudopods proves that there must be an active, rearward movement of a cellular structure attached to the bead independent of cell translocation (Fig. 2b, d).

The convergent and parallel paths followed by beads on Ax2 and *mhcA*⁻ cells, respectively, illustrate the role of myosin II in the rearward transport mechanism of Ax2 cells and indicate

that capping and myosin II-independent rearward transport are separable processes. The difference in trajectories also explains the patterns of bead redistribution shown in Fig. 1. The detectable rearward transport of beads but not soluble conA on mutant cells⁵ may be due to greater membrane glycoprotein crosslinking to beads, which could increase the probability of linkage to the cytoskeleton.

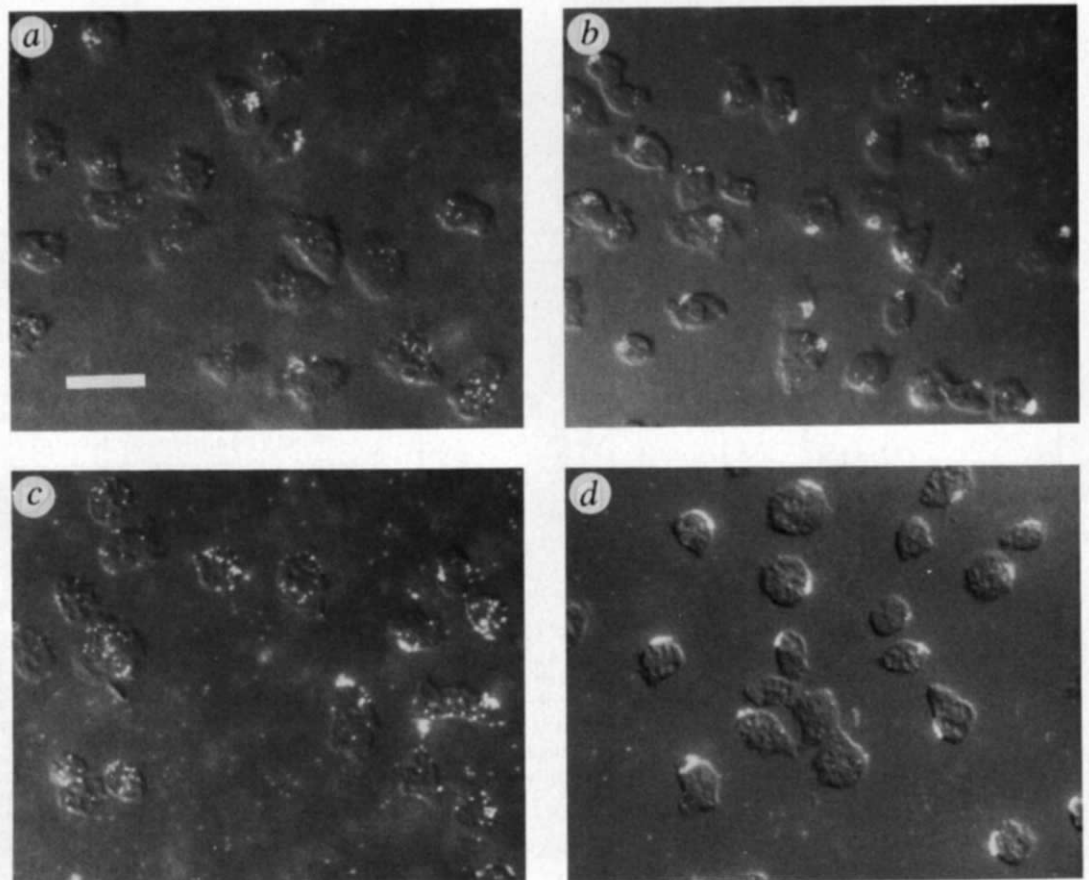
Differences in velocity also distinguish transport on *mhcA*⁻ and wild-type cells. The average velocity of bead transport on *mhcA*⁻ cells, $2.59 \pm 0.25 \mu\text{m min}^{-1}$ (mean $\pm$ s.e.m.), is significantly less than that on Ax2 cells, $4.55 \pm 0.25 \mu\text{m min}^{-1}$ ($P < 0.001$, two-tailed Student *t*-test; Fig. 3). The *mhcA*⁻ mutant's slower rearward transport velocity may explain, in part, the decreased chemotactic velocity of a *Dictyostelium* mutant with a nonfunctional, truncated myosin II heavy chain⁶. The occurrence of rearward transport on stationary cells indicates, however, that the surface transport mechanism is not sufficient for cellular translocation. Additional factors, such as the strength and rate of formation of contacts with the substratum, are likely to be important.

The observed difference in the average velocities of bead transport results from faster transport at the middle and rear of wild-type cells (Fig. 3). At their leading edges, the velocities of rearward transport on Ax2 and *mhcA*⁻ cells are similar. As myosin II increases the velocity of bead transport behind the leading edge, one of its functions in motility may be to increase the magnitude and distance over which a tractional force is exerted to advance the cell.

Mechanical changes that occur during capping of soluble conA in wild-type but not *mhcA*⁻ cells further characterize the

FIG. 1 ConA-coated beads are randomly distributed on Ax2 (a, b) and *mhcA*⁻ (c, d) cells 5 min after addition (a, c) but after 20 min (b, d), many cells have transported the beads rearward. On Ax2 cells (b) the beads are in clusters similar to conA caps. The beads on *mhcA*⁻ cells (d) are aligned along the rear edge of the cell. Beads on the sides of cells mark the previous rear edges of cells that have changed their direction of locomotion. The beads are transported on the external, dorsal surface of the cell, as determined by focusing through the cell and double-labelling with rhodamine-avidin of beads coated with biotinylated conA on fixed cells (data not shown). Superficial beads can be distinguished from internalized ones which have a rapid, saltatory motion characteristic of organelles and vesicles. Sodium azide, a metabolic poison that inhibits capping¹⁸, also inhibits bead transport on *mhcA*⁻ cells, indicating ATP dependence (data not shown). Scale bar, 20 μ m.

METHODS. Ax2 and *mhcA*⁻ cells, plated on glass coverslips in HL5 medium the day before an experiment, were washed with and incubated in MES buffer⁵ for 1 h. Although the cells had been starved for 1 h, bead transport occurs on cells starved for up to 8 h. ConA-coated, 490-nm diameter Fluoresbrite carboxylate beads (Polysciences), prepared



as described¹⁷, were placed on the cells for 60 s. Excess beads were aspirated and MES buffer was added to cover the cells. Cells were observed using a Zeiss IM-35 microscope and $\times 40$ objective with simultaneous differential interference contrast and epifluorescence illumination.

role of myosin II in rearward transport. During capping, wild-type cells initially stiffen and then relax as capping proceeds. The *mhcA*⁻ cells neither stiffen nor cap under the same conditions⁵. Stiffening has been attributed to isometric tension generated in the membrane-associated cytoskeletal cortex by myosin II, soon after the initiation of capping. Consistent with the cortical flow hypothesis⁹, stiffness diminishes as the cortex is drawn up a tension gradient towards the rear of the cell. Cross-linked membrane glycoproteins associated with the cortical cytoskeleton are carried along to form the cap. The convergent bead paths on Ax2 cells support such a model, in which myosin II concentrated at the posterior of the cell¹⁰ generates a tension gradient that pulls the actin cytoskeleton and attached membrane proteins rearwards. It is unclear how far forward the resultant tractional force is transmitted in the cell as there is no distinct point on the cell at which myosin II begins to affect the velocity of transported beads.

The similar bead transport velocities at the leading edges of Ax2 and *mhcA*⁻ cells and the inhibition of both by cytochalasin suggest that the same myosin II-independent, actin-based mechanism is responsible in each case. For example, one or more myosin I (single-headed) species may provide the myosin II-independent force¹¹. A correlation of the velocity data with the locations of myosins I and II in *Dictyostelium* supports this hypothesis: myosin I is concentrated at the leading edge of the cell¹² and myosin II at the rear¹⁰. Recently, several myosin I null mutants have been produced which have defects in motility^{13,14} (M. Titus, personal communication). It will be interesting

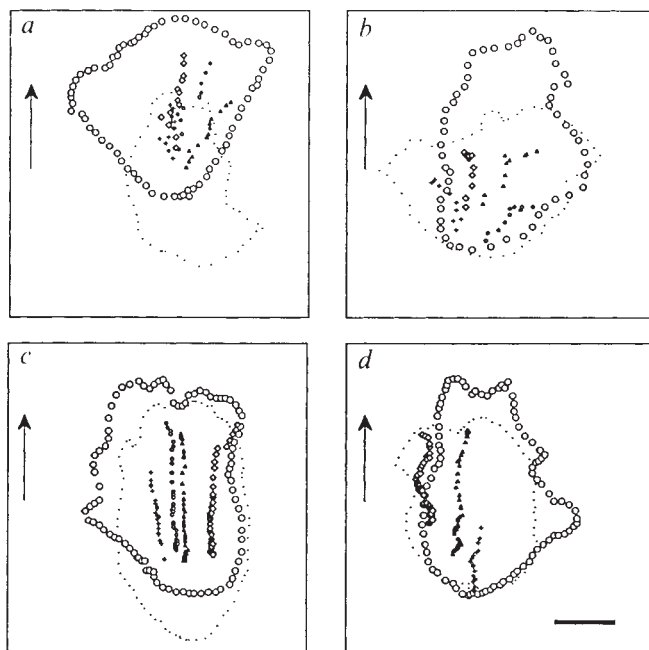


FIG. 2 Time-lapse recordings of bead transport on Ax2 (a, b) and *mhcA*⁻ (c, d) cells demonstrate rearward transport and the different paths followed by beads. Representative examples are shown of pathways on translocating Ax2 (a) and *mhcA*⁻ cells (c) and stationary Ax2 (b) and *mhcA*⁻ (d) cells with pseudopodial activity. Although not depicted, the beads on these cells were ultimately transported to the rear edge. Bead positions are plotted every 10 s. Arrows show the direction of cell motility; dotted, initial cell outline; circles, final cell outline. Time between initial and final cell outlines: (a) 130, (b) 90, (c) 570, (d) 910 s. Scale bar, 5 μ m.

METHODS. Bead transport on single cells was observed using a $\times 40$ phase contrast, water-immersion objective and recorded using an ISIT camera (Dage-MTI) and time-lapse sVHS recorder (Panasonic). The positions of beads and a reference point on the rear edge of the cell were measured every 10 s using software for a Series 151 image processor (Imaging Technology, Woburn, Massachusetts). Bead paths are plotted in the cell's reference frame and superimposed on the final cell outline. The error in measuring bead position was $\pm 0.2 \mu$ m (95% confidence).

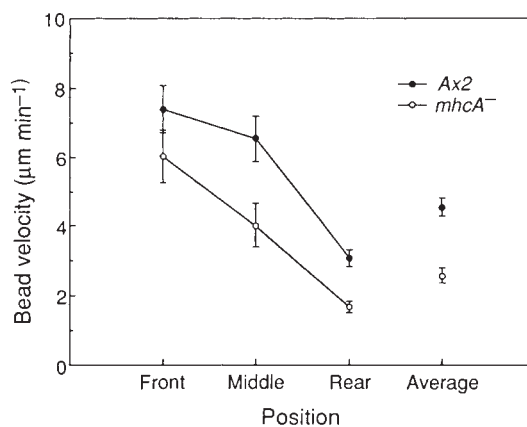


FIG. 3 Bead transport velocities on Ax2 and *mhcA*⁻ cells. On both cell types the velocity of a bead generally decreases as it moves rearward. The difference in initial velocities on Ax2 and *mhcA*⁻ cells is neither significant nor reproduced in a separate experiment. The differences in the middle, rear and average velocities are significant ($P < 0.01$, < 0.001 and < 0.001 , respectively). Bead velocities are relative to the cell's reference frame; Bars represent s.e.m. For the front, middle, rear and average velocities, $n = 19$ each for Ax2 and $n = 20, 19, 17$ and 19 for *mhcA*⁻.

METHODS. Rearward-transported beads that started within 1μ m of the leading edge of the cell were tracked until they had reached their final position at the rear edge. Velocities were measured over $1-2 \mu$ m at the front and rear edges of the cell immediately after a bead had started moving or before it had stopped, respectively, and at the middle of a bead's migration. The average velocity is defined as the distance between the initial and final points of transport at the leading and trailing edges of the cell, divided by the elapsed time. Data were collected from a consecutive series of 74 Ax2 and 78 *mhcA*⁻ single-cell observations. A second series of 41 Ax2 and 40 *mhcA*⁻ cells yielded similar results. The number of tracked beads is less than the number of cells because of the low probability of a bead falling within 1μ m of the leading edge.

to see if there are particular defects in rearward transport at the leading edges in these mutants. Actin polymerization and consequent actin flow may also contribute to the myosin II-independent transport. Polymerization does not drive rearward transport on neuronal growth cones³ but may on fish keratinocytes¹⁵.

Single-particle tracking experiments on moving cells have characterized both forward¹⁶ and rearward^{2,17} transport of membrane proteins. Comparison of wild-type and *mhcA*⁻ mutant amoebae now reveals two distinct mechanisms of rearward transport. The observation that particle transport and cell locomotion both occur more rapidly in wild-type than mutant cells suggests that myosin II-dependent and -independent mechanisms both contribute to motility in wild-type amoebae. Similar analyses of rearward transport in myosin I null mutants may help us to understand the contribution of these actin-based motors to cell motility. □

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Calcium channel characteristics conferred on the sodium channel by single mutations

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THE sodium channel, one of the family of structurally homologous voltage-gated ion channels¹, differs from other members, such as the calcium and the potassium channels, in its high selectivity for Na⁺. This selectivity presumably reflects a distinct structure of its ion-conducting pore. We have recently identified two clusters of predominantly negatively charged amino-acid residues, located at equivalent positions in the four internal repeats of the sodium channel as the main determinants of sensitivity to the blockers tetrodotoxin and saxitoxin². All site-directed mutations reducing net negative charge at these positions also caused a marked decrease in single-channel conductance². Thus these two amino-acid clusters probably form part of the extracellular mouth and/or the pore wall of the sodium channel. We report here the effects on ion selectivity of replacing lysine at position 1,422 in repeat III and/or alanine at position 1,714 in repeat IV of rat sodium channel II (ref. 3), each located in one of the two clusters, by glutamic acid, which occurs at the equivalent positions in calcium channels. These amino-acid substitutions, unlike other substitutions in the adjacent regions, alter ion-selection properties of the sodium channel to resemble those of calcium channels. This result indicates that lysine 1,422 and alanine 1,714 are critical in deter-

mining the ion selectivity of the sodium channel, suggesting that these residues constitute part of the selectivity filter of the channel.

Figure 1 shows an alignment of the amino-acid sequences in the regions encompassing the short segment SS2 of the four repeats of sodium³⁻⁷ and calcium⁸⁻¹⁰ channels. The positions corresponding to one of the two amino-acid clusters that determine the toxin sensitivity of the sodium channel are occupied by glutamic acid (E in one-letter notation) in all four repeats of calcium channels. At these positions of sodium channels, repeats I and II have negatively charged residues but repeat III has a positively charged residue (Lys 1,422), and repeat IV an uncharged residue (Ala 1,714). These differences in charge in the regions suggested to form part of the channel lining^{2,11-17} led to the idea that they might underlie the difference in the ion selectivity of sodium and calcium channels. We therefore examined ion permeation properties of mutated sodium channels in which Glu is substituted for Lys 1,422 (K1422E), for Ala 1,714 (A1714E), or both (K1422E·A1714E).

Figure 2 shows a series of whole-cell current records from *Xenopus* oocytes injected with messenger RNA specific for the wild-type rat sodium channel II, the mutant K1422E or the mutant A1714E, using one of Na⁺, Li⁺, K⁺ or NH₄⁺ as the only external monovalent cation (similar data for Rb⁺ and Cs⁺ not shown). Whereas no inward current was detected with K⁺, Rb⁺ or Cs⁺ for the wild-type or the mutant channel A1714E, these ions carried current through the mutant K1422E; increased inward current was observed with NH₄⁺ compared with the other cations.

To investigate ion selectivity more quantitatively, we used the macro-patch technique¹⁸, which allows accurate measurement of current responses at defined ion compositions on both sides of the membrane (Fig. 3). The resulting peak current-voltage relationships showed that the reversal potential, measured with high external Na⁺ and high internal K⁺ concentration, was +41.9 ± 1.4 mV for the wild type (mean ± s.d., n = 7, n indicating

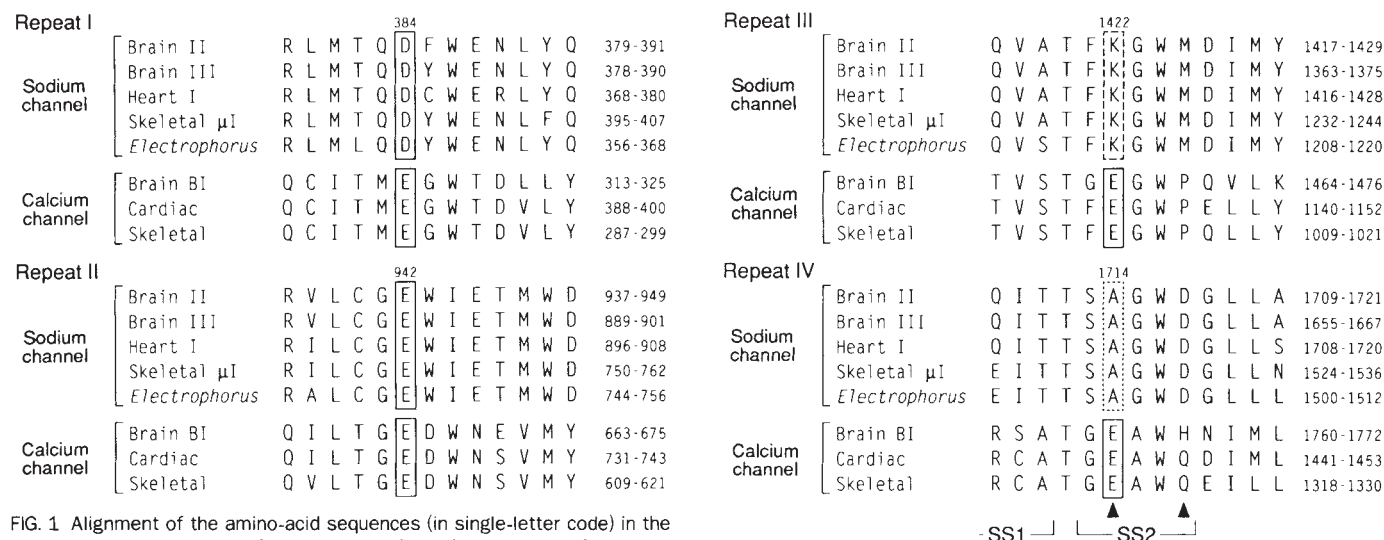


FIG. 1. Alignment of the amino-acid sequences (in single-letter code) in the regions encompassing the SS2 segment of the four repeats of different sodium and calcium channels. The sequences are as follows: rat brain sodium channel II (brain II; ref. 3); rat brain sodium channel III (brain III; ref. 5); rat heart I sodium channel (heart I; ref. 7); rat skeletal muscle μ I sodium channel (skeletal μ I; ref. 6); *Electrophorus electricus* electroplax sodium channel (ref. 4); rabbit brain calcium channel BI (brain BI; ref. 10); rabbit cardiac dihydropyridine (DHP)-sensitive calcium channel (cardiac; ref. 9); and rabbit skeletal muscle DHP-sensitive calcium channel (skeletal; ref. 8). The numbers of the amino-acid residues in each sequence are given on the right-hand side. The positions of the SS1 and SS2 segments¹⁷ are indicated at the bottom, and those of the two amino acid clusters identified as determinants of sensitivity to tetrodotoxin and saxitoxin² are indicated by arrowheads. The cluster that contains the residues identified as determinants of ion selectivity is boxed (negatively charged residues, solid lines; positively

charged residues, dashed lines; uncharged residues, dotted lines). The number of the residues in this cluster of sodium channel II are given. Rat brain sodium channel I (ref. 3) has the same amino-acid sequence as sodium channel II in the regions shown. A putative *Drosophila* sodium channel²⁴ has Glu(E) at the position corresponding to Lys 1,422 of brain II.

METHODS. Construction of plasmids carrying cDNAs encoding the wild-type (pRII-2A; ref. 25) and single mutant channels has been described^{2,11,12}. The pSP65 (ref. 26) recombinant plasmid pAM(K1422E·A1714E), encoding the double mutant channel, was constructed by replacing the ~1.8-kilobase-pair *Bst*III-*Sal*I fragment from pAM(K1422E) by the corresponding fragment from pAM(A1714E) (ref. 2). mRNAs specific for the wild-type and mutant channels were synthesized as described².

the number of patches), $+6.8 \pm 0.8$ mV ($n=9$) for the mutant K1422E, and $+30.1 \pm 1.3$ mV ($n=4$) for the mutant A1714E. The calculated permeability ratio P_K/P_{Na} was 0.03 for the wild type, 0.69 for K1422E and 0.15 for A1714E, which indicates that these mutant channels have lost the high ion selectivity for Na^+ over K^+ of the wild-type channel. Furthermore, current records of the mutant channels showed marked outward rectification, whereas macroscopic channel gating properties of the mutants were not significantly altered as judged by the time courses of channel activation and inactivation. This suggested that external Ca^{2+} might block these mutant channels much more potently than the wild-type channel^{19,20}.

To test this idea, we studied the effect of changing external Ca^{2+} concentration on whole-cell current in oocytes expressing

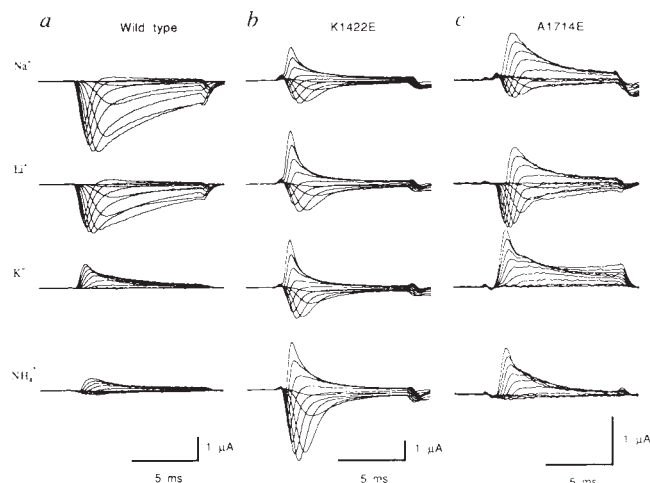


FIG. 2 Whole-cell currents recorded from *Xenopus laevis* oocytes expressing the wild-type rat sodium channel II (a), the mutant K1422E (b) and the mutant A1714E (c) with external solutions containing different monovalent cations. Each column of current traces was from a single oocyte. Whole-cell currents were measured with a two-electrode voltage clamp²⁷, using depolarizing pulses ranging from -40 to $+50$ mV in steps of 10 mV from a holding potential of -80 mV. The external solution contained 115 mM of the test cation as chloride salt, 1.8 mM $CaCl_2$ and 10 mM HEPES (pH 7.2 with the corresponding hydroxide). The external solution was changed by a slow perfusion system in the order shown. The sequence of maximum peak inward current amplitudes was as follows: for wild type, $Na^+ > Li^+ \gg NH_4^+ > K^+ \approx Rb^+ \approx Cs^+$; for K1422E, $NH_4^+ > Na^+ \approx Li^+ \approx K^+ \approx Rb^+ \approx Cs^+$; and for A1714E, $Li^+ > Na^+ \gg NH_4^+ > K^+ \approx Rb^+ \approx Cs^+$. Because of the unknown concentration of Na^+ and K^+ in the oocyte, these sequences give no quantitative estimate of the ion selectivity of the channels. K^+ influx through A1714E was not seen, presumably because it was masked by efflux of internal Na^+ and K^+ (see Fig. 3 for K^+ permeability). Other mutant channels carrying amino-acid substitutions in SS2 and adjacent regions of repeats I–IV were also tested for ion selectivity: for repeat I, R379Q, Q383E, Q383K, D384E, D384N, W386Y, E387Q, E387S, E387Y, N388R and Q391K; for repeat II, E942Q, W943Y, E945Q and E945K; for repeat III, M1425Q, M1425K, D1426N, D1426K and D1426K; and for repeat IV, D1717N, D1717Q and D1717K (ref. 2). These mutant channels showed no significant alterations in ion selection properties, judged by the ratio of whole-cell maximum peak inward current measured with various monovalent cations to that measured with Na^+ . An exception was that the mutant E387Y, whose single-channel conductance for Na^+ is reduced more than 100-fold (ref. 2), showed more than ten times larger inward current with Li^+ than with Na^+ . The permeability ratio P_K/P_{Na} and the sensitivity to divalent cations of this mutant channel could not be determined reliably because of the very low conductance. METHODS. Oocytes were injected with 30 – 50 nl mRNA ($0.5 \mu\text{g } \mu\text{l}^{-1}$) and incubated for 4 – 7 days²⁷. All electrophysiological experiments were performed at 19 – 21°C . Current records were sampled at 10 kHz after low-pass filtering at 3 kHz (-3 dB). The traces shown have been corrected for leakage and capacitive transients using a P/6 correction method. Because of insufficient time resolution using a two-electrode voltage clamp, artefactual biphasic current responses were observed near the reversal potential, indicating that about 2 ms is required for the membrane potential to settle. This value, however, is short enough for estimating peak inward current.

the mutant channel K1422E. As the Ca^{2+} concentration in the external medium decreased, the inward current became larger (Fig. 4a). When Na^+ in external medium was entirely replaced by Ca^{2+} or Ba^{2+} , large inward currents were observed, indicating that the mutant K1422E is highly permeable to Ca^{2+} and Ba^{2+} (Fig. 4b). Figure 4c shows the dependence on external Ca^{2+} concentration of maximum peak inward current carried by a series of ion mixtures, in which Ca^{2+} concentration is progressively raised as Na^+ concentration is reduced. Na^+ influx was blocked as the external Ca^{2+} concentration increased. At high Ca^{2+} concentrations, larger inward currents were observed, indicating that Ca^{2+} itself becomes the main charge carrier. This anomalous dependence is very similar to that observed for native calcium channels^{21–23}, although the IC_{50} value (the concentration inhibiting maximum peak inward current by 50%) of the mutant channel K1422E ($74 \pm 4 \mu\text{M}$; $n=5$) was higher by two orders of magnitude than those of native calcium channels. Na^+ influx through the mutant channel A1714E was also blocked by external Ca^{2+} , with $IC_{50} = 119 \pm 18 \mu\text{M}$ ($n=3$). No permeation of Ca^{2+} or Ba^{2+} was detected, however, for A1714E or for the wild-type channel (data not shown), which showed a weak block by Ca^{2+} ($IC_{50} = 8.4 \pm 1.0$ mM; $n=6$).

The blocking potency of other divalent cations was tested in external medium without added Ca^{2+} . Whereas the IC_{50} values of the wild-type channel were 1.9 ± 0.2 mM ($n=3$) for Zn^{2+} , 2.7 ± 0.7 mM ($n=3$) for Cd^{2+} , and 2.2 ± 0.2 mM ($n=3$) for Co^{2+} , the mutant channel K1422E showed much higher sensitivity: IC_{50} $3.1 \pm 1.2 \mu\text{M}$ ($n=4$) for Zn^{2+} , $7.4 \pm 2.1 \mu\text{M}$ ($n=3$) for Cd^{2+} , and $13 \pm 3 \mu\text{M}$ ($n=3$) for Co^{2+} . The mutant channel A1714E showed intermediate sensitivity: IC_{50} $97 \pm 17 \mu\text{M}$ ($n=3$) for Zn^{2+} , $210 \pm 10 \mu\text{M}$ ($n=3$) for Cd^{2+} , and $220 \pm 20 \mu\text{M}$ ($n=3$) for Co^{2+} .

When the mutations K1422E and A1714E were combined, the resulting double-mutant channel (K1422E·A1714E) showed sensitivity to Ca^{2+} even higher than that of the single mutant channels, with an IC_{50} of $5.4 \pm 1.4 \mu\text{M}$ ($n=4$) (Fig. 4d). Inward current was strongly reduced at $500 \mu\text{M}$, but increased inward current was observed with increased Ca^{2+} concentrations and the mutant K1422E·A1714E was also permeable to Ba^{2+} . From the IC_{50} value, we estimate that inward current is carried mainly by Ca^{2+} even at $500 \mu\text{M}$. We were unable to test block of this mutant channel by Zn^{2+} , Cd^{2+} and Co^{2+} because Ca^{2+} chelators bind these cations more strongly than Ca^{2+} and because this

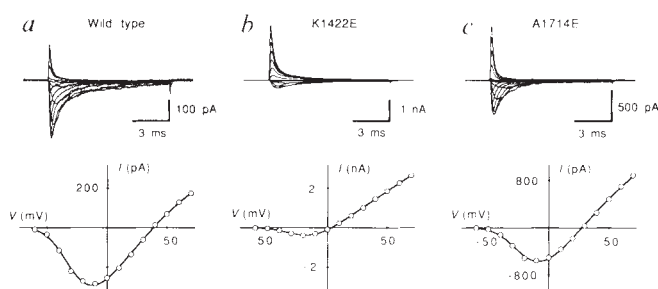
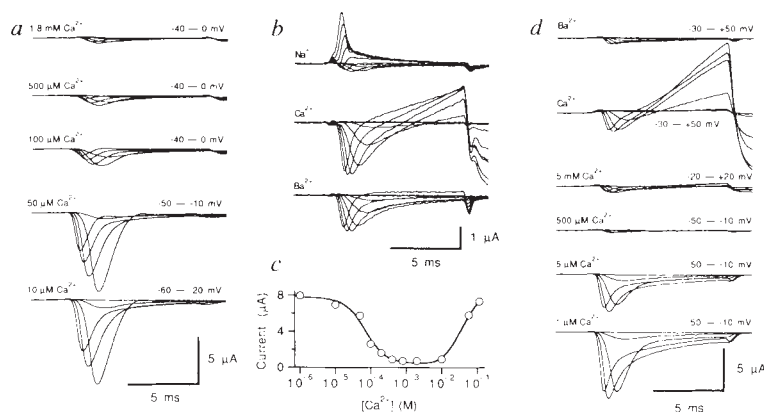


FIG. 3 Currents recorded from inside-out macro-patches excised from oocytes expressing the wild-type sodium channel II (a), the mutant K1422E (b) and the mutant A1714E (c). Upper panel: current traces in response to depolarizing pulses ranging from -60 to $+70$ mV in steps of 10 mV from a holding potential of -100 mV. Lower panel: peak current-voltage relationships. Continuous lines represent the non-linear least-square fits according to the m^3 kinetics of activation¹⁸ and the Goldman-Hodgkin-Katz current equation^{28,29}. METHODS. The pipette solution contained 100 mM NaCl, 16 mM KCl, 1.8 mM $CaCl_2$ and 10 mM HEPES (pH 7.2 with NaOH). The bath solution contained 100 mM KCl, 16 mM NaCl, 1.8 mM EGTA and 10 mM HEPES (pH 7.2 with KOH). Current records were sampled at 40 kHz after low-pass filtering at 10 kHz (-3 dB), and corrected using a P/8 method. The permeability ratio P_K/P_{Na} was calculated by the Goldman-Hodgkin-Katz voltage equation^{28,29} without taking permeation of Ca^{2+} into account.

FIG. 4 Effects of external Ca^{2+} on whole-cell currents from oocytes expressing the mutant channels K1422E (a–c) or K1422E–A1714E (d). a, Series of current traces of K1422E in external solution which contained the indicated concentration of CaCl_2 , 115 mM NaCl and 10 mM HEPES (pH 7.2 with NaOH). Depolarizing pulses ranged between the indicated potentials in steps of 10 mV from a holding potential of -80 mV. b, Current traces of K1422E with normal frog Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl_2 and 10 mM HEPES, pH 7.2 with NaOH; upper traces), with a solution containing 115 mM CaCl_2 and 10 mM HEPES, pH 7.2 with $\text{Ca}(\text{OH})_2$ (middle traces) and with a solution containing 100 mM BaCl_2 and 10 mM HEPES, pH 7.2 with $\text{Ba}(\text{OH})_2$ (lower traces). Depolarizing pulses ranged from -40 to $+50$ mV in steps of 10 mV from a holding potential of -80 mV. The inward currents were not affected by replacing external Cl^- with methanesulphonate (data not shown). The large outward current and the tail current observed in the presence of high external Ca^{2+} resulted from Ca^{2+} influx activating an endogenous chloride channel in the oocyte^{30,31}; this component was blocked efficiently by addition of 500 μM niflumic acid³² (data not shown). c, Plot of maximum peak inward current through K1422E as a function of external Ca^{2+} concentration, when the total concentration of Ca^{2+} plus Na^+ was kept constant at 115 mM. The external solution contained the indicated concentration of CaCl_2 , the remaining concentration of NaCl and 10 mM HEPES (pH 7.2 with NaOH). Maximum peaks inward current was obtained from a complete peak current-voltage relationship and no correction was made for shifts in the voltage-dependence of channel activation. The initial part of the curve was drawn according to $y = 1/(1 + (x/\text{IC}_{50})^n)$ with IC_{50} of 71 μM and a Hill coefficient (n) of 1.4; the right part was drawn by eye. IC_{50} values for divalent cations



(Ca^{2+} , Zn^{2+} , Cd^{2+} and Co^{2+}) were obtained with external solutions that contained varying concentrations of the test cation as chloride salt, 115 mM NaCl and 10 mM HEPES (pH 7.2 with NaOH). d, Current traces of K1422E–A1714E obtained at a high Ba^{2+} or Ca^{2+} concentration as in b followed by a series of current traces obtained with decreasing concentrations of external Ca^{2+} as in a. Depolarizing pulses ranging between the indicated potentials in steps of 10 mV from a holding potential of -80 mV. In a, c and d, the Ca^{2+} concentration was buffered with EGTA up to 1 μM , with nitrilotriacetic acid between 5 and 100 μM , and was unbuffered above 100 μM . No Ca^{2+} chelators were added to external medium containing Zn^{2+} , Cd^{2+} or Co^{2+} .

channel is blocked by residual Ca^{2+} present in external medium without Ca^{2+} chelators.

Accumulating evidence supports the view that the SS1–SS2 region of voltage-gated ionic channels forms part of the channel lining^{2,11–17}. Single amino-acid substitutions in the region of a potassium channel have been shown to enhance permeability to larger cations, NH_4^+ and Rb^+ , but not to alter the ability of this channel to exclude Na^+ (ref. 15). Our work reveals that the single mutations K1422E and A1714E in the SS2 segment of repeats III and IV alter the ion selectivity of the sodium channel to resemble those of calcium channels: decreased selectivity for Na^+ and block by Ca^{2+} and other divalent cations at micromolar concentrations. The mutant channel K1422E is even permeable to Ca^{2+} and Ba^{2+} . Furthermore, the channel carrying both mutations is not only permeable to Ca^{2+} and Ba^{2+} , but also is selective for Ca^{2+} over Na^+ at their physiological concentrations. These results indicate that Lys 1,422 and Ala 1,714 are critical in distinguishing the sodium channel from the calcium channel with respect to ion selectivity. They also suggest that these sites of the sodium channel and corresponding sites of the calcium channel form part of the selectivity filter of these channels. □

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HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides

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THE mutant human cell line T2 is defective in antigen presentation in the context of class I major histocompatibility complex (MHC) molecules^{1,2}, and also in that transfected T2 cells show poor surface expression of exogenous human class I (HLA) alleles³. Both defects are thought to lie in the transport of antigenic peptides derived from cytosolic proteins into the endoplasmic reticulum (ER)^{1,2}, as peptide-deficient class I molecules might be expected to be either unstable or retained in the ER⁴. The products of several mouse class I (*H-2*) genes, and the endogenous gene *HLA-A2* do, however, reach the surface of T2 cells at reasonable levels although they are non-functional^{3,5,6}. We report here that, as expected, poorly surface-expressed HLA molecules do not significantly bind endogenous peptides. Surprisingly, H-2

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molecules expressed in T2 also lack associated peptides, arguing that 'empty' complexes of mouse class I glycoproteins with human β_2 -microglobulin are neither retained in the ER nor unstable. HLA-A2 molecules, however, do bind high levels of a limited set of endogenous peptides. We have sequenced three of these peptides and find that two, a 9-mer and an 11-mer, are derived from a putative signal sequence (of IP-30, an interferon- γ -inducible protein⁷), whereas a third, a 13-mer, is of unknown origin. The unusual length of two of the peptides argues that the 9-mers normally associated with HLA-A2 molecules may be generated before their transport from the cytosol rather than in a pre-Golgi compartment. To our knowledge, this is the first report of the isolation of a fragment of a eukaryotic signal peptide generated *in vivo*.

T2 cell-surface expression levels of the transfected mouse and human MHC genes are presented in Table 1; HLA-A3 is not detectable on the surface, whereas H-2D^p and H-2K^b are expressed at levels comparable to those seen on transfected control cells (T0, C1R). Peptides associated with the MHC molecules genes were isolated⁸ and characterized by reverse phase high-performance liquid chromatography (HPLC). The HLA-A3-associated peptide profile from the control, C1R cells transfected with HLA-A3 (C1R/A3, Fig. 1a; see Table 1 for abbreviations) has multiple peaks. The HLA-A3-profile from T2/A3, however, shows a diminished peptide content (Fig. 1b). This low level of peptide binding correlates with the absence of HLA-A3 cell-surface expression and the observation that HLA-A3 molecules in T2/A3 do not acquire endoglycosidase H resistance (and thus are not transporting through the cis/medial Golgi) despite association with β_2 -microglobulin (β_2 m; E. Click and P.C., manuscript in preparation). The H-2D^p- and H-2K^b-associated peptide profiles in wild-type cells (Fig. 1c, e), like the control HLA-A3-associated profile, show multiple peaks. The H-2D^p-associated profile from T2/DP (Fig. 1d) remains at background levels. This was also true when T2/DP cells were labelled with a mixture of all tritiated amino acids (data not shown). The peptides associated with H-2K^b from T2/K^b were also greatly reduced (Fig. 1f). Thus, unlike human HLA-A3 molecules, murine H-2D^p and H-2K^b molecules are expressed on the surface of T2 cells at levels

similar to those found in wild-type cells (Table 1), despite a reduced level of associated peptides.

Endogenous HLA-A2 is the only human class I allele with appreciable surface expression on T2 and the similarly defective .174 cell line (20–50% of parental cell levels^{9,10}). HLA-A2-associated peptides isolated from the parental cell T0 (Fig. 2a) yield ≥ 13 peaks. The peptide profile of HLA-A2 purified from T2, in contrast, shows very high levels of three dominant peaks (Fig. 2b). The peptides do not associate with unbound HLA-A2

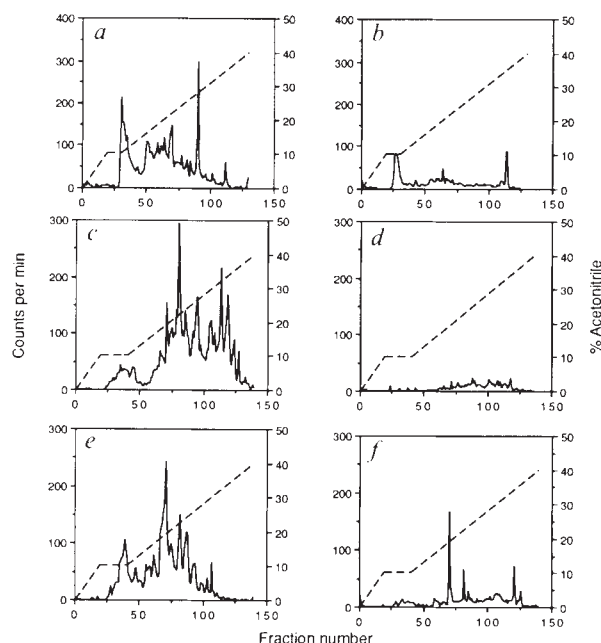


FIG. 1 HPLC analysis and comparison of transfected class I-associated radiolabelled peptides from T2 and wild-type cells. Class I molecules were affinity-purified from $1-5 \times 10^9$ of the following cells: HLA-A3 from a, C1R/A3 and b, T2/A3; H-2D^p from c, T0/DP and d, T2/DP; H-2K^b from e, C1R/K^b and f, T2/K^b.

METHODS. Stable transformed cell lines of T0, T2, and C1R expressing H-2D^p or H-2K^b have been described³ and C1R and T2 transformed cell lines expressing HLA-A3 were similarly established. Cells were pelleted, washed and labelled with L-[3,4,5-³H] leucine and L-[4,5-³H] lysine (NEN) for 6–8 h, 37 °C, at 10^7 cells ml⁻¹, in Leu-free, Lys-free RPMI-1640 (Gibco) supplemented with 3% dialysed fetal calf serum, and 0.1 mg ml⁻¹ gentamicin (Gibco). Cells were pelleted and stored at –20 °C. Class I-associated peptides were isolated using the protocol described by Van Bleek and Nathenson⁸, with minor modifications. Pellets were lysed at 10^7 cells ml⁻¹ in 2% polyoxyethylene(9) lauryl ether (Sigma), 10 mM Tris, pH 7.4, 150 mM NaCl, 0.02% Na₃, 0.5 mM PMSF, 0.1 mM (TLCK), 5 mM iodoacetamide. After pelleting nuclei, EDTA was added to 5 mM, lysates were cleared by centrifuging for 1 h at 36,000 r.p.m. and applied to affinity columns, washed and eluted²⁹. Eluates were denatured by adding 10% acetic acid and boiling for 3 min; low-M_r species were separated from class I heavy chain and β_2 m by filtering eluates through a Centricon 10 (Amicon). Filtrates (containing material of <M_r 10 K) were analysed on a reverse-phase column (μ Bondapak C₁₈, Waters), using a Waters HPLC system. Gradients were generated using an increasing concentration of acetonitrile in 0.1% trifluoroacetic acid. Flow rate was 1 ml min⁻¹, and 0.5 min fractions were collected and counted with Aquasol2 (NEN) in a liquid scintillation counter (LKB). A mixture of unlabelled peptides (20 nmol each of malaria circumsporozoite 378–398, influenza B nucleoprotein 82–94, influenza A nucleoprotein 384–394, and insulin) was included in every sample analysed by HPLC and absorbance monitored at 214 nm to ensure the reproducibility of each separation. Affinity columns were packed with Biogel A15m beads (BioRad) to which had been coupled the following monoclonal antibodies: a, b, GAP.A3 (ref. 25), anti-HLA-A3; c, d, 7-16.10 (ref. 23), anti-H-2D^p; e, f, 28-8-6S (ref. 24), anti-H-2K^b. One-dimensional PAGE analyses of each of the class I glycoprotein eluates showed the presence of β_2 m as well as heavy chains (data not shown). Pairs of profiles for each allele were normalized for recovery of labelled class I molecules.

TABLE 1 Cell-surface expression of transfected MHC class I alleles on T2 and control cells

Exp. 1	Cell line	anti-H-2D ^p	control
	T0	16	6
	T0/DP	393	7
	T2	7	42
	T2/DP	373	11
Exp. 2		anti-H-2K ^b	control
	C1R	21	20
	C1R/K ^b	742	21
	T2	37	72
	T2/K ^b	617	13
Exp. 3		anti-HLA-A3	control
	C1R	2	4
	C1R/A3	386	3
	T2	4	25
	T2/A3	6	8

Cells were stained as previously described³ and 5,000 cells were analysed on an Ortho Cytofluorograf; fluorescence was measured on a linear scale and values are reported as mean fluorescence channel. Staining antibodies were: 7-16.10 (ref. 23) (anti-H-2D^p); 28-8-6S (ref. 24) (anti-H-2K^b); and GAP.A3 (ref. 25), (anti-HLA-A3). Negative control staining antibodies were: experiment 1 and 2, GAP.A3; experiment 3, 7-16.10. The addendum to the cell line name indicates that cell line has been transfected with an MHC allele: D^p, H2-D^p; K^b, H2-K^b; A3, HLA-A3.

molecules following solubilization, as lysis of T2 cells in the presence of unlabelled influenza matrix protein-derived peptide M58-68 (Fig. 2*d*), which is known to bind HLA-A2¹¹, results in the loss of only one minor peak compared with a control extract (Fig. 2*c*). The total amount of peptide associated with T2-derived HLA-A2 is significantly greater than that associated with T2-derived HLA-B5 (Table 2). Radioactivity in the low-relative-molecular-mass (M_r) fraction recovered from HLA-B5 was comparable to that recovered from the transferrin receptor, a molecule not expected to bind peptides, and no peaks were detectable when this material was subjected to HPLC (data not shown). This result was also obtained when HLA-B5 was purified from T2 cells labelled with ³H-Phe, -Pro and -Tyr in addition to ³H-Leu and -Lys (data not shown). Low- M_r radioactivity isolated from HLA-A2, however, was 20 times higher than background with either labelling protocol.

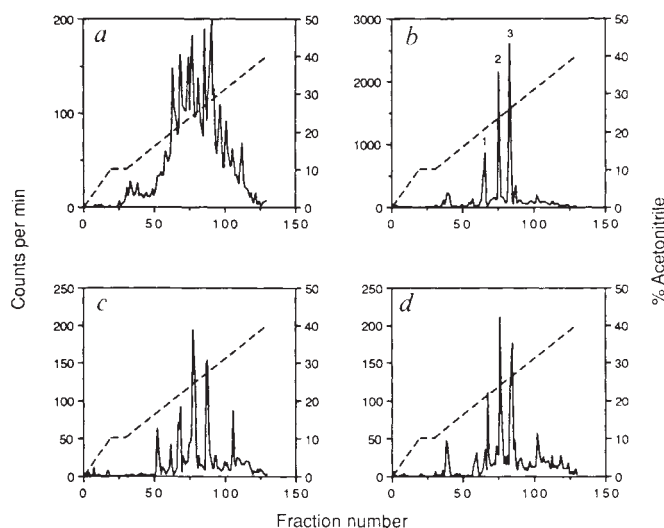
The three dominant HLA-A2-associated peptides in T2 were sequenced (Fig. 2*e*). Peptide 1, a truncated version of peptide 2, is the nonamer expected for HLA-A2-associated peptides¹². Comparison with the reported consensus sequence motif for HLA-A2-associated peptides¹² reveals that it contains the predicted leucine dominant anchor residue at position 2 and the predicted dominant valine residue at position 9. Peptides 2 and 3 are an 11-mer and 13-mer, respectively. A homology search revealed no proteins containing the sequence of peptide 3. Peptides 1 and 2 are identical in sequence to the C terminus of

the predicted hydrophobic signal sequence of IP-30, a interferon- γ -inducible protein constitutively present in haematopoietic cells⁷. Thus, HLA-A2 binds peptides that are presumably present in the ER independently of the putative peptide transporter molecules whose genes are deleted in T2 (refs 13-15).

One explanation for the differential transport properties of human MHC molecules, such as HLA-A3 and HLA-B5, and mouse molecules such as H-2D^p and H-2K^b, in T2 is that 'empty' human molecules are less stable than 'empty' mouse class I molecules associated with human β_2 m. An alternative hypothesis is that HLA class I molecules are specifically retained in the ER until they are released by the action of an HLA-linked *trans*-acting gene, deleted in .174 and T2 (ref. 16). The transport defect in the related cell line .134 results from the inactivation of the *PSF-1/RING 4* gene¹³, which encodes a member of the ATP-binding-cassette family of membrane transporters. This gene is deleted in .174 and T2 (refs 13-15), suggesting that the central defect is the decreased supply of cytosolic peptides and that peptide binding is the key step required to release the human class I molecules for transport. H-2 class I molecules may not be susceptible to the retention mechanism and therefore do not have to bind peptides to be transported out of the ER.

Class I MHC molecules are generally associated with non-amer peptides, or octamers in the case of H-2K^b (refs 8, 12, 17). Peptides longer than nine amino acids can bind H-2D^p from T2 *in vitro*, but have ~100-fold faster off-rates than the nonameric peptide¹⁸. HLA-A2 in .174 is less stable than in wild-type cells¹⁹, and we have similarly found that the half-life of cell-surface HLA-A2 molecules is decreased on T2 (M.W. and P.C., manuscript in preparation). These results suggest that the longer peptides bound by HLA-A2 in T2 may have a lower affinity than those in wild-type cells. Incubation of HLA-A2-restricted peptides with .174 and T2 cells increases cell-surface expression of HLA-A2, and their inclusion in lysates of .174 and T2 cells facilitates the immunoprecipitation of HLA-A2- β_2 m dimers⁵. Presumably the added peptides replace dissociated endogenous peptides and stabilize HLA-A2 molecules which would otherwise decay^{4,18}.

Two of the sequenced peptides associated with HLA-A2 from T2 are longer than the canonical nine amino acids. The generation of nonamers is therefore likely to be intimately connected with the normal antigen processing mechanism. This might



e Peptide sequences

1. L L D V P T A A V
2. L L L D V P T A A V Q
3. V/G L F R G G P R G L L A V

FIG. 2 HPLC analysis of HLA-A2-associated peptides. HLA-A2 was affinity purified from *a*, 10^9 T0/D^p cells, *b*, 10^9 T2/D^p cells, *c*, 2×10^8 T2/D^p cells lysed in the absence of M58-68 (see below), *d*, 2×10^8 T2/D^p cells lysed in the presence of 100 μ M unlabelled M58-68, *e*, Sequences of HLA-A2-associated peptides from T2.

METHODS. HLA-A2 was purified over a BB7.2 (anti HLA-A2, ref. 27) monoclonal antibody affinity column. One-dimensional PAGE analyses of eluates showed the presence of β_2 m as well as heavy chain (data not shown). HLA-A2-associated peptides were isolated and analysed as in Fig. 1. M58-68 is a synthetic peptide (synthesized by Multiple Peptide Systems) corresponding to amino acids 58-68 of the influenza matrix protein, which has been shown to bind specifically to HLA-A2 (ref. 11). For sequence analysis, peptides were isolated from 2×10^{10} cells and automated Edman degradation was carried out in an Applied Biosystems Model 477A Sequencer with on-line phenylthiohydantoin analysis using an Applied Biosystems Model 120A HPLC apparatus. Yields at the proposed C-terminal cycle for peptides 1, 2 and 3 were 4.9, 21.9 and 6.1 pmol, respectively. The predicted lengths of the peptide are therefore presented with a high degree of confidence.

TABLE 2 Recoveries of affinity-purified molecules and associated peptides

Allele	Cell line	Total recovery (c.p.m. $\times 10^{-5}$)	Peptide recovery (c.p.m. $\times 10^{-3}$)	% Peptide associated (c.p.m.)
HLA-A2	T0/D ^p	5.9	7.1	1.2
HLA-A2	T2/D ^p	2.3	6.3	2.8
HLA-A2	T2/K ^b	3.3	8.0	2.4
HLA-A2	T2/A3	4.9	8.3	1.7
HLA-B5	T2/D ^p	12.0	1.5	0.1
HLA-B5	T2/K ^b	14.0	1.4	0.1
HLA-B5	T2/A3	13.0	2.4	0.2
Tf-Rc	T0/D ^p	12.0	1.2	0.1
Tf-Rc	T2/D ^p	6.0	1.8	0.3
Tf-Rc	T2/A3	6.6	0.8	0.1
Tf-Rc	C1R	2.8	0.4	0.1

The amount of peptide-associated radioactivity as a fraction of total class-I-associated radioactivity is increased for HLA-A2 purified from T2, compared to HLA-B5 from T2. Cells were labelled and lysed as in Fig. 1. Class I glycoproteins and the transferrin receptor (Tf-Rc) were affinity-purified by applying cell lysates to the following monoclonal antibody columns connected in tandem: IG12 (anti-Tf-Rc; ref. 26), BB7.2 (anti-HLA-A2; ref. 27), and w6/32 (anti-HLA class I; ref. 28). No molecules other than HLA-B5 were detected in the w6/32 eluate on electrophoretic analysis (data not shown). Associated peptides were separated and analysed as described in Fig. 1. Reported recoveries are normalized for 10^9 cells.

involve cytosolic proteolysis, perhaps by proteasomes (genes related to which are linked to the MHC²⁰⁻²²), followed by peptide transport into a pre-Golgi compartment. The peptides found at such high levels associated with HLA-A2 from T2 (Fig. 2b) are not readily apparent in wild-type HLA-A2 (Fig. 2a). Therefore competition by the cytosolically derived pool must be highly effective and as peptides 1 and 2 are derived from a signal peptide, normal peptide binding may occur in the ER itself. Because peptides 2 and 3 are an 11-mer and 13-mer, respectively, nonamers in wild-type cells could be generated before transport into the ER. However, genes encoding 'trimming proteases'¹² may be deleted in T2, and direct evidence that the IP-30-derived peptides bind to HLA-A2 in the ER is lacking. The putative signal peptide for IP-30 is 37 amino acids long⁷, and the region associated with HLA-A2 contains an aspartic acid residue. The length of the peptide may affect its processing such that it is unusually long-lived. In addition, the charged aspartic acid may prevent integration of the epitope peptides into the lipid bilayer, a property that could increase their half-life as well as making them accessible for binding to HLA-A2 molecules. □

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Experimental evidence for an alternative to directed mutation in the *bgl* operon

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THE directed mutation hypothesis¹⁻⁶ suggests that some mutations occur more often when selectively advantageous than when neutral or disadvantageous, challenging the principle that the selective value of a mutation does not affect the rate of its occurrence⁷⁻¹¹. Mutations in the *bgl* operon of *Escherichia coli* have been reported to be a case of directed mutation². *E. coli* K12 strain χ 342LD cannot grow on salicin but derivatives with two mutations in the *bgl* operon, an excision of *IS150* (formally called *IS103*; ref. 12) from *bglF* and a point mutation or insertion in *bglR*¹³⁻¹⁵, grow rapidly on this sugar. When χ 342LD is grown on a medium containing salicin, *bglF* excision mutants accumulate to a frequency of >1%, even though these mutants are reportedly² unable to grow on salicin, and *Sal*⁺ double mutants subsequently attain a high frequency. Comparable accumulations of excision mutants and *Sal*⁺ double mutants are not observed in the absence of salicin. As salicin is not mutagenic, it has been suggested that excision mutations in *bglF* might serve only to create the potential for a secondary selectively advantageous mutation². We show here, however, that these double mutants can be accounted for by spontaneous mutation to intermediate genotypes in non-growing populations, coupled with slow growth of some of these intermediates on salicin, which enables their populations to reach a size where secondary mutations allowing rapid growth on salicin become common.

After confirming Hall's² observation that *Sal*⁺ double mutants are produced at a high frequency when χ 342LD is grown on MacConkey-salicin (MS) agar (Fig. 1), we further investigated whether excision mutants or other intermediate genotypes could grow on the salicin in MS agar, as suggested elsewhere⁸. We

did not follow previously reported experimental procedures² that purported to rule out such growth, for two reasons. First, these procedures could have inhibited the growth of the mutant cells on MS agar. Second, only one excision mutant (XSW1) was tested for growth on salicin. Other independent excision mutants (or phenotypically similar intermediates) might be able to grow on salicin.

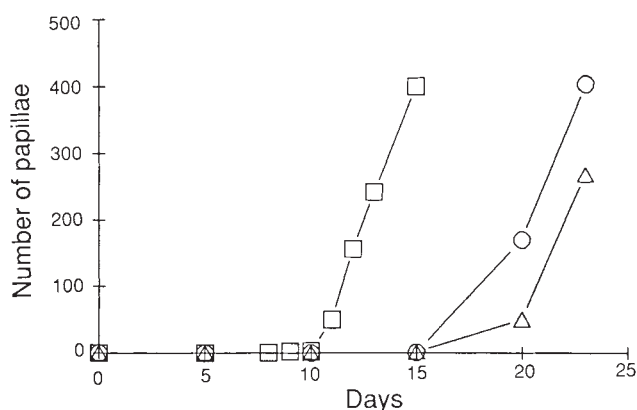


FIG. 1 The cumulative number of papillae appearing on MS plates incubated at 30 °C. Strain χ 342LD was grown overnight in L broth² at 30 °C, $\sim 7 \times 10^8$ cells were spread onto each plate, where they grew into a lawn of $\sim 10^{11}$ cells in 24 h. Papillae that overgrew the lawn were scored by streaking cells on MS agar and observing the resulting colonies for 10 days. On MS indicator agar, *Sal*⁺ double mutants produce red colonies, whereas excision mutants produce white colonies that then give rise to red papillae in 2-4 days²; χ 342LD also produces white colonies, but these do not give rise to red papillae until ~ 10 days. ○, Papillae containing numerous excision mutants but no detectable *Sal*⁺ double mutants; △, presence of *Sal*⁺ double mutants in the papillae; □, small white papillae which were neither excision mutants nor *Sal*⁺ double mutants. (These small white papillae are another type of mutant that is capable of growing on substances, other than salicin, present in old plates.) Each point is the mean of four replicates. Similar results were obtained (data not shown) when using the plating procedure previously described². Although in the previous study excision mutants and *Sal*⁺ double mutants were seen earlier than in our experiments (using either procedure), our experiments confirm that these genotypes achieve substantial numbers when strain χ 342LD is incubated for a prolonged period on MS agar.

Instead, we spread ~ 100 cells of each of five different intermediate genotypes together with $\sim 10^9$ $\chi 342$ LD cells onto MacConkey plates with and without salicin. One of the intermediates tested (XSW1) was the same excision mutant studied previously²; the other four were isolated by us. All five intermediates form white colonies, but produce red papillae (out-growths) in 2–4 days, on MS agar; this is the criterion previously used² to define excision mutants in order to monitor their accumulation. Four of the intermediates, including XSW1, overgrew the $\chi 342$ LD lawns to form ~ 100 papillae in 9 days on MS plates (Fig. 2), but none formed any papillae on MacConkey plates without salicin. We also saw no papillae in 9 days on control MS plates spread with $\chi 342$ LD only (Fig. 2; see also Fig. 1), indicating that the papillae seen on the MS plates seeded with four of the five intermediates were not derived from the background lawn. To verify that the papillae that overgrew the lawn during the first 9 days on MS plates resulted from growth by intermediates rather than secondary Sal^+ mutants, we sampled cells from papillae as they overgrew the lawn. Most of these cells formed white colonies that produced red papillae in 2–4 days on MS agar, indicating that they were the seeded intermediates rather than Sal^+ double mutants. The papillae on the seeded MS plates thus resulted from growth by the intermediates on the salicin in MS agar. There were striking differences among the intermediates in their ability to grow on MS agar, even though all five seem to be *bglF* excision mutants on the basis of the colony-phenotypic criterion².

These results contradict Hall's claim that excision (or excision-like) mutants cannot grow on the salicin in MS agar, and they therefore undermine his inference that these mutations do not occur in the absence of salicin. This inference was based on the fact that no excision-like mutants were found among $\sim 3,600$ cells taken from old colonies on MacConkey plates without salicin (versus $>1\%$ on MS plates)². But, if some of these mutants can grow on the salicin in MS agar, as shown above, then the presence of so many mutants in old colonies on MS plates can be accounted for by a small number of mutational events, making it necessary to test not thousands, but hundreds of millions of cells from non-selective plates to determine whether these mutations also occur in the absence of salicin.

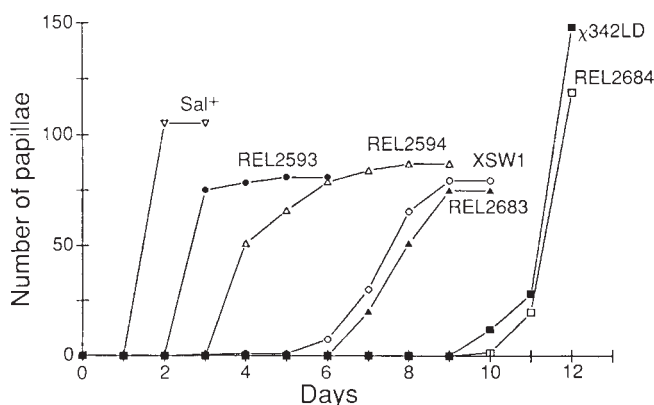


FIG. 2 The cumulative number of seeded mutants forming papillae on MS plates incubated at 30 °C. Cells (~ 100) of each of five independently isolated intermediates (REL2593, REL2594, REL2683, REL2684, XSW1) and one Sal^+ double mutant were plated together with $\sim 7 \times 10^8$ $\chi 342$ LD cells. As a control, we plated $\sim 7 \times 10^8$ $\chi 342$ LD cells without any seeded mutants. The majority of papillae overgrowing the lawn on plates seeded with REL2593, REL2594, REL2683 and XSW1 were the seeded intermediates. By contrast, most of the papillae that overgrew the lawns on the plates seeded with REL2684 and the $\chi 342$ LD control plates were the small white type (squares, Fig. 1). Each point is the mean of two replicates. Analysis of variance of the time required for 50% of the seeded intermediates to appear as papillae indicates heterogeneity among the five intermediate genotypes in their growth properties on MS agar ($F = 155.4$, $P < 0.001$).

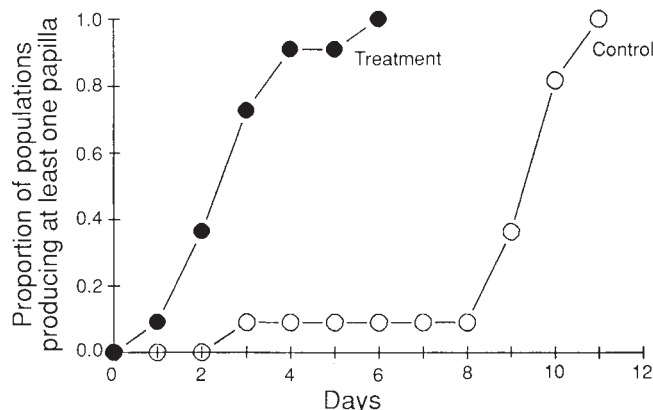


FIG. 3 The cumulative percentage of MS plates, spread with cells from control (○) and treatment (●) populations, producing at least one papilla. Eleven treatment populations were allowed to accumulate mutants in the absence of salicin for 15 days, and were then re-grown in rich medium (also without salicin) for one day. Eleven control populations were grown in rich medium for one day, but starting from a genetically homogeneous isolate. To generate each treatment population, we inoculated $\chi 342$ LD into 10 ml L broth, which was incubated at 30 °C; each population rapidly grew to its stationary-phase density of $\sim 7 \times 10^9$ cells ml^{-1} . The population remained in this medium without any additional nutrients for 15 days, during which time the density of viable cells decreased to $\sim 2 \times 10^8$ cells ml^{-1} . We then transferred 0.1 ml of the surviving cell population into 10 ml of fresh L broth for one day. These steps yielded a treatment population in which mutations had been allowed to accumulate for 15 days, but whose density and physiological state were equivalent to a control population that was grown for one day in L broth starting with $\chi 342$ LD cells taken from a single colony. We spread 0.1 ml from treatment and control populations on MS plates, which were then monitored for papillae that overgrew the background lawn during incubation at 30 °C. Most papillae that overgrew the lawns derived from control populations were neither excision mutants nor Sal^+ double mutants, but instead were the small white type observed after comparable periods in the experiment shown in Fig. 1. By contrast, almost all papillae that overgrew the lawns derived from treatment populations comprised either excision mutants or Sal^+ double mutants.

To demonstrate that excision-like mutations do occur spontaneously, non-growing populations of $\chi 342$ LD were allowed to accumulate mutations for 15 days in the absence of salicin. These treatment populations were then re-grown overnight in fresh medium (still without salicin) so that their densities and physiological states were equivalent to control populations grown overnight starting from single-colony isolates. Samples from 11 treatment and 11 control populations were then spread onto MS plates, which were monitored for papillae that overgrew the background lawn. In every case, the treatment populations produced one or more papillae in 6 days, whereas the control populations (with one exception) did not produce papillae until 9 days (Fig. 3). The median time for first papilla appearance was 3 days for the treatment populations and 10 days for the controls; this difference is highly significant (Mann-Whitney $U = 115.5$, $P < 0.001$). Excision, or excision-like, mutations happen frequently in populations that are starved but not exposed to salicin.

Our experiments demonstrate that excision-like mutations occur spontaneously in the absence of salicin and that many of the resulting mutants can grow on the salicin in MS agar, albeit more slowly than 'true Sal^+ ' double mutants. These mutants produce papillae on MS agar that contain millions of cells, thereby increasing the frequency of Sal^+ double mutants by many orders of magnitude relative to salicin-free control plates, much as Symonds speculated⁸. There is no reason to invoke directed mutation in this system.

This is our second investigation¹⁶ of purported cases of directed mutations. In both cases, proponents of the directed mutation hypothesis^{1,2} detected certain mutants only after incubation

on selective media, and they inferred that the selective agents had increased the appropriate mutation rates by many orders of magnitude. But both previous studies failed to detect two critical processes: the accumulation of mutants in non-growing populations, even in the absence of the selective agents thought to cause the mutations, and growth by certain genotypes on the selective media, which increased the number of cells susceptible to mutation relative to the control media. These and other physiological and ecological processes¹⁷⁻²¹ must be convincingly excluded in apparent cases of directed mutation. □

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Structure of the DNA-binding domain of zinc GAL4

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THE yeast transcriptional activator GAL4 binds co-operatively to four related 17-base-pair sequences within an upstream activating sequence (UAS_G) to activate transcription of the GAL1 and GAL10 genes¹. It belongs to a class of gene regulatory proteins which all contain a highly conserved cysteine-rich region within their DNA-binding domains^{2,3}. This region binds zinc⁴⁻⁷ and it has been proposed that the cysteine residues coordinate the zinc, creating a structure analogous to one of the 'zinc fingers' of the transcription factor TFIIIA (ref. 8). Using ¹H-¹³Cd two-dimensional nuclear magnetic resonance spectra of the cadmium form of the domain, we previously showed that the protein contains a Cd₂Cys₆ cluster where cysteines 11 and 28 act as bridging ligands⁹. A similar study of a fragment of GAL4 has recently been published¹⁰. We report here the solution structure of the DNA binding domain of GAL4; two helix-turn-strand motifs pack around a Zn₂Cys₆ cluster in a novel pseudo-symmetrical arrangement. The results show that the GAL4 zinc-binding domain differs significantly from both the TFIIIA-type zinc finger¹¹ and the steroid hormone receptor DNA-binding domains¹².

We initially studied several different fragments of the GAL4 protein containing the cysteine-rich region (Fig. 1). Gel retardation assays showed that a fragment consisting of residues 1-54 binds specifically to a consensus DNA binding site. Two-dimensional (2D) ¹H NMR spectra of this fragment, however,

contained a severely overlapped region, indicative of random coil structure. We had shown that, following α -chymotryptic digestion of a larger fragment of GAL4 (residues 1-147), the major stable fragment consisted of residues 5-49. For structure determination, we expressed and purified GAL4 (7-49). The 2D ¹H NMR spectra of this fragment, which binds nonspecifically to DNA, suggested that we had removed most of the poorly structured regions of the protein, facilitating assignment of the spectra and identification of the secondary structure¹³. The chemical shifts and patterns of nuclear Overhauser effect (NOE) interactions in spectra of GAL4(7-49) and GAL4(1-54), excluding the badly overlapped region, are very similar, strongly suggesting that the structured region within the two fragments is identical.

Residues 10-40 form a well-defined domain of volume $\sim 16 \times 20 \times 20 \text{ \AA}$. No long-range NOEs were observed involving residues 7, 8 or 42-49, and it is likely that they are mobile in this protein fragment (Fig. 2). The structure contains two helix-turn-strand motifs between Cys 11 and Lys 23 and between Cys 28 and Tyr 40 (Fig. 3). These motifs pack around the Zn₂Cys₆ cluster and the sidechains of Leu 19 and Trp 36 (see below), so that the negative charge of the zinc cluster is partially offset by the helix dipoles. The two motifs are related to each other by a twofold pseudo-symmetry axis which passes between the two helices and through the centre of the zinc cluster (Fig. 3). Although only the cysteine residues are conserved between the two motifs, the symmetry of the polypeptide fold is one of the most striking features of the structure of this DNA-binding domain. Comparing residues 11-23 with 28-40, the r.m.s. deviation of the α atoms is 0.80 $\text{\AA}$.

Residues 24-27 form a turn, with a *cis* Lys 25-Pro 26 bond linking the two motifs. This may explain the strong conservation of Pro 26 in this family of proteins (Fig. 1). If Pro 26 is changed to Leu in GAL4 the protein requires a higher level of zinc to retain activity and, presumably, correct folding⁴. The domain is compact, but a channel through to the Zn₂Cys₆ cluster may

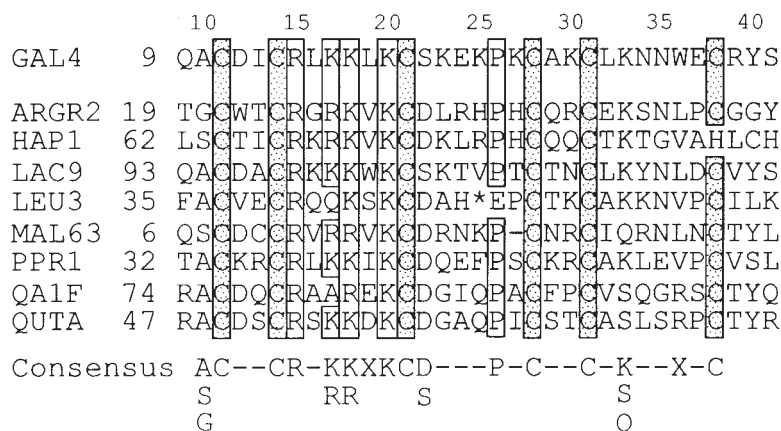


FIG. 1 Alignment of nine related fungal gene regulatory proteins listed by gene name (adapted from ref. 3). The sequence numbering at the top refers to GAL4. The letter X in the consensus sequence represents a hydrophobic residue. In the LEU3 sequence, the asterisk represents insertion of the sequence ERAP (Glu-Arg-Ala-Pro).

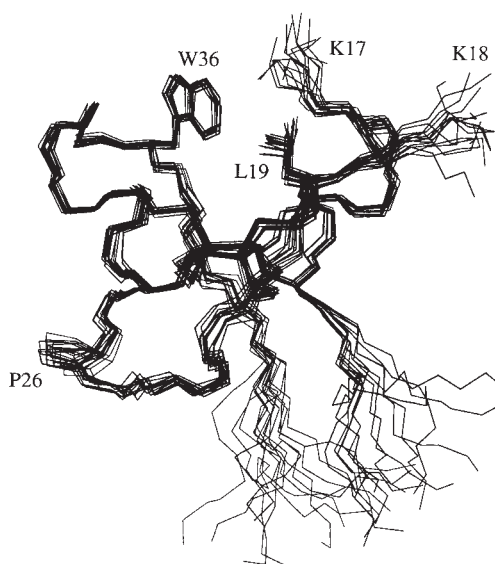


FIG. 2 Superimposition of 15 structures (arbitrarily chosen from the 30 computed) showing the backbone of residues 7-43, several side chains, the 6 cysteine residues and the 2 zinc atoms making up the Zn_2Cys_6 cluster. METHODS. Comparison of spectra run under slightly different conditions, such as varying temperature, using the program ANSIG¹⁹ together with our previously reported complete assignment¹³ enabled us to assign most of the crosspeaks in the NOE spectra. The effects of spin diffusion were assessed by analysis of the Taylor transform of NOE spectra recorded with mixing times of 30, 60, 120 and 200 ms. A method similar to that proposed by Hyberts and Wagner²⁰ was used. NOE connectivities derived from cross-peaks seen to result significantly from spin diffusion were assumed to be

due to protons ≤ 6 Å apart. NOE connectivities from other strong, medium or weak crosspeaks were assumed to be due to protons ≤ 2.5 , ≤ 3.2 and ≤ 5.0 Å apart, respectively. The structure is based on a total of 567 NOE connectivities, including 171 sequential, 83 medium range and 149 long range NOEs. Sequential NOEs refer to neighbouring residues. Medium and long range NOEs are defined as (i) to $\leq(i+4)$ and (i) to $\geq(i+5)$ respectively. Coupling constants $^3J_{\text{HN}\alpha}$ were estimated from splittings in the $\text{NH}-\text{C}\alpha\text{H}$ crosspeaks in DQF-correlation (COSY) spectra²¹ whereas $^3J_{\alpha\beta}$ coupling constants were from the $\text{C}\alpha\text{H}-\text{C}\beta\text{H}$ crosspeaks in an E. COSY spectrum²². We obtained ϕ dihedral angle constraints for seven residues and stereo-specific assignments and χ_1 torsion angle constraints for another seven residues including four of the cysteines²³. Further constraints came from three hydrogen bonds, the NH protons of which exchanged slowly and were identified in the α -helices of the initial structures. Constraints based on the cysteine-metal ion connectivities identified in the cadmium domain⁹ were included on the basis of identical DNA binding activity in the zinc and cadmium forms of the protein⁵ and of similar patterns seen in NOE spectra. Electrospray ionization mass spectrometry showed that the cadmium and zinc forms of the protein have identical metal ion stoichiometry (data not shown). The sulphur-zinc bond distances and tetrahedral coordination were derived from the EXAFS and X-ray absorption near-edge structure (XANES) data which suggest an all-sulphur coordination when GAL4(7-49) is purified under the conditions used for preparation of samples for NMR studies (ref. 5, and our unpublished results). Thirty structures were computed using the X-PLOR program and the YASAP protocol²⁴. The structures are well-defined with an atomic r.m.s. distribution about the mean coordinate position of 0.35 Å for the backbone atoms and 0.91 Å for all heavy atoms within the region of residues 10-40. There are no violations of the NOE constraints of >0.5 Å. The stereochemistry of the structures, as judged by the ϕ and ψ angles in a Ramachandran plot and by energy calculations, is good. A Lennard-Jones van der Waals energy (E_{LJ}) of -103 ± 5 kcal mol⁻¹ was calculated using the CHARMM force field²⁵. Structures computed without any of the zinc constraints were very similar (data not shown). The coordinates will be deposited in the Brookhaven Protein Data Bank. This drawing and those in Fig. 3 were generated using the program MOLSCRIPT²⁶.

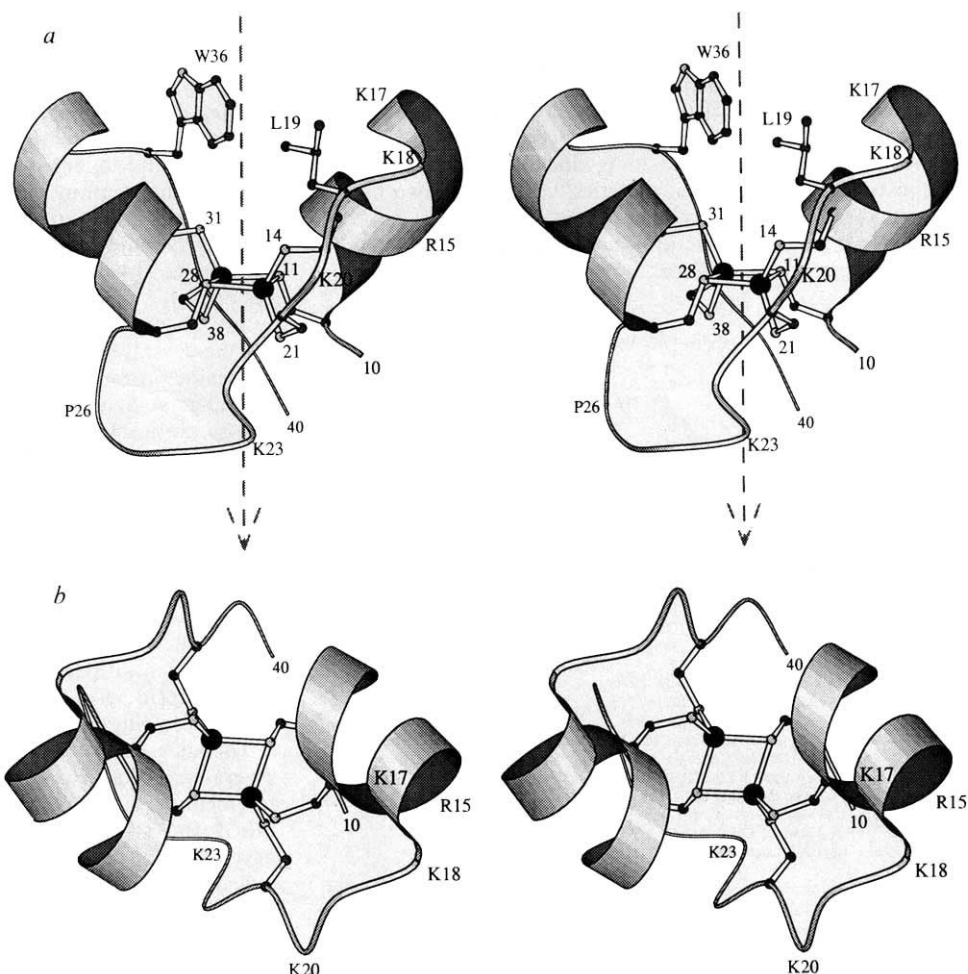


FIG. 3 Stereo views of the GAL4 DNA-binding domain showing the Zn_2Cys_6 cluster and the location of residues discussed in the text. In *a* the structure is shown in the same orientation as in Fig. 2. The 2-fold symmetry axis is indicated by the arrow. In *b* a view down the 2-fold symmetry axis is shown. The α -helices lie between Cys 11 and Lys 18 and between Cys 28 and Asn 35; the extended strands run from Leu 19 to Lys 23 and from Trp 36 to Tyr 40. There are several hydrogen bonds between the polypeptide backbone and the zinc cluster: HN (Cys 11) to $\text{S}\gamma$ (Cys 21), HN (Cys 14) to $\text{S}\gamma$ (Cys 11) and the symmetry-related HN (Cys 28) to $\text{S}\gamma$ (Cys 38) and HN (Cys 31) to $\text{S}\gamma$ (Cys 28).

explain why the zinc can be so rapidly replaced by cadmium⁹. Four of the slowly exchanging amide protons form hydrogen bonds to sulphur atoms in the Zn₂Cys₆ cluster, stabilising it. This bonding is seen in other zinc proteins including the glucocorticoid receptor DNA binding domain¹⁴ and metallothionein¹⁵. EXAFS (extended X-ray absorption fine structure) spectra have suggested the presence of an oxygen or nitrogen ligand bound to zinc in GAL4 in certain buffers⁵; moreover, if Cys 21 is replaced by Ala, GAL4 still binds DNA sequence specifically, albeit with reduced affinity¹⁶. The hydroxyl of Tyr 40 is close to the zinc ions (~4.5 Å) and, with a small conformational change, it might displace one of the Cys ligands.

The structure of GAL4 helps rationalize the patterns of conserved residues among the family of fungal gene regulatory proteins (Fig. 1) and suggests some details of DNA binding. The symmetry-related residues at positions 19 and 36 are almost always hydrophobic; when they are not, as in proteins QA1F or QUTA, either an Arg-Glu or Arg-Asp pair occurs, and may form a salt bridge. Basic residues at positions 15, 17, 18 and 20 are conserved across the class, indicating that they may make interactions with DNA. It seems that Lys 23 is required for sequence-specific recognition¹⁷. The residue immediately before Cys 11 is always small; in the HAP1 protein, changing residue 10 to Arg selectively abolishes DNA-binding to UAS1 (ref. 3). A long side chain at this position in the structure would have to project outwards between residues 15 and 20, interfering with DNA binding. These results, together with the knowledge that residues 49-54 are also important for sequence-specific DNA binding (ref. 17 and our own results) suggest that the amino-terminal helix-turn-strand motif and the carboxy-terminal region of the domain are involved in DNA binding. Presumably, residues within the region 41-54 become structured on binding to DNA.

This study shows that the DNA-binding domain of GAL4 belongs to a class of zinc domains that is distinct from both the TFIIIA-type zinc finger, which has Cys₂His₂ zinc coordination, and the steroid hormone receptor DNA-binding domain in which the zinc coordination is Cys₄. The TFIIIA-type zinc finger contains an α -helix and a β -hairpin sandwiched round a zinc ion¹¹, whereas the steroid hormone receptor DNA-binding domain consists of a double loop-zinc-helix motif¹⁸ which contains two separate ZnCys₄ moieties¹². In GAL4, two helix-turn-strand motifs pack symmetrically around the Zn₂Cys₆ cluster to form a novel structure, the mode of binding to DNA must also therefore be different. □

Solution structure of the DNA-binding domain of Cd₂-GAL4 from *S. cerevisiae*

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THE GAL4 protein activates transcription of the genes required for galactose utilization in *Saccharomyces cerevisiae*¹. The protein, consisting of 881 amino acids, is dimeric when bound to one of the approximately twofold symmetrical DNA sites present in the galactose upstream activating sequence (UAS_G)²⁻⁵. Here we use two-dimensional NMR spectroscopy to determine the structure of an amino-terminal fragment of GAL4 (residues 1-65). This fragment, a monomer in solution, binds as a dimer specifically to UAS_G-containing DNA. Residues 9-40 form a well defined, compact globular cluster, whereas residues 1-8 and 41-66 show considerable conformational mobility in the absence of DNA. The compact domain contains a motif in which six cysteines, located on two symmetrically related helix/extended strand units connected by a long loop, coordinate two central zinc ions, forming a bimetal-thiolate cluster⁶⁻¹¹. The zincs were replaced by NMR-active ¹¹³Cd in most of our work and structural parameters are therefore derived from the Cd₂-protein. The structure obtained for the GAL4 DNA-binding domain represents a novel DNA-binding motif. Essentially the same conformation is observed for the compact domain in solution using NMR techniques as was seen for the central core of the N-terminal fragment bound to DNA using crystallographic techniques¹². Thus, the core of the DNA-binding domain changes little upon binding DNA.

Assignments for proton and ¹¹³Cd NMR resonances were obtained and interproton nuclear Overhauser enhancement (NOE) and coupling constant data were then used to obtain a three-dimensional structure for the Cd(II) substituted DNA-binding domain using standard methods¹³. Only a central core of residues 9-40 forms a compact globular domain. The terminal residues 1-8 and 41-65 show little persistent structure in solution. There are many inter-residue NOE crosspeaks observed for the central core (30 per residue, on average). Almost all of the amide protons of the central core (except Ser 22 and Glu 24) exchange sufficiently slowly with the bulk solvent (at pH 7.0) that saturation techniques used for H₂O solvent suppression do not affect amide proton intensity. The amide protons of the flexible tails are in more rapid exchange with solvent, indicating solvent accessibility and a less-ordered structure¹⁴. Only a few weak NOE crosspeaks were observed for protons in the disordered region. Some NOE crosspeaks between adjacent amino acids were seen for residues 6-9 and 40-43, indicating that amino acids next to the core of the DNA-binding domain are somewhat less mobile than the extreme N and C termini, but that they do not pack against the central protein core. In addition, the residues outside the core region have narrow resonance lines, indicating increased mobility.

The diagonal map of NOE crosspeaks (Fig. 1) highlights several secondary structural features of Cd(II) GAL4(65), the fragment we have used, in agreement with those observed for the Zn(II) substituted protein¹⁵. The stretch of *i, i+3* connectivities for residues 12-18 and 30-35 shows the presence of two α -helices. Although the structural integrity of the protein would seem to be provided mainly by the way in which the two

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central metal ions are liganded, some hydrophobic packing is observed with the side chain of Trp 36 (by residues Ile 13, Leu 16, Leu 19, Lys 30 and Lys 31) and with the side chain of Tyr 40 (by residues Gln 9 to Asp 12, Lys 23 and Lys 25 to Lys 27). Our proton NMR assignments, pattern of NOE crosspeak connectivities, and secondary structural element designations generally agree with those previously published¹⁵ for the Zn(II) substituted protein (residues 7–49), but not with those published¹⁶ for Cd(II) GAL4 (residues 1–62).

The distance geometry program DG-II (ref. 17) was used with 208 lower distance bounds, 608 upper distance bounds and 40 ϕ and χ_1 torsion angle constraints to calculate a set of 25 structures for GAL4(65). The torsion angles from the resulting structures are given in Fig. 2. The depiction of the structure with the smallest number of distance violations (the largest violation was 0.3 Å) in Fig. 3 shows that the axes of the two α -helices, residues 12–17 and 29–34, respectively, form an angle of approximately 90°. They are connected by a loop containing an extended chain from residues 19–23 and a tight bend at *cis* Pro 26. The slowly exchanging amide protons for the GAL4 DNA-binding domain can be attributed to hydrogen bonds: Cys 28(NH)–Glu 24(O), Asp 12(NH)–Arg 39(O), Leu 19(NH)–Cys 14(O) and possibly Cys 11(NH)–Tyr 40(O η). The remainder of the slowly exchanging amides (Cys 14, Arg 15, Cys 31

and Leu 32) are involved in α -helices and form hydrogen-bonds to the carbonyl oxygen of the *i*-3rd or *i*-4th residue. Residues Lys 18 and Asn 35 adopt the left-handed helical conformation normally reserved for Gly with positive ϕ and ψ torsion angles. These are the C-terminal cap residues for helices 1 and 2, respectively.

The apparent internal repeat in the Cys-(X)₂-Cys-(X)₆-Cys-(X)₆-Cys-(X)₂-Cys-(X)₆-Cys motif is reflected in the torsion angles of residues 10–22 and 27–39 (Fig. 2). Each of these sequences forms a similar α -helix/tight-turn/extended-chain structure. A striking characteristic of the packing for these motifs is revealed by comparing the protein with itself after rotation by 180° such that the backbone of residues 10–22 is superimposed onto the backbone of residues 27–39, and vice versa (Fig. 4a). The root-mean-square residual deviation of the atoms is 1.3 Å, showing internal 2-fold symmetry. Although only the cysteine residues are conserved in an amino-acid sequence alignment between these two segments, the implication is that the

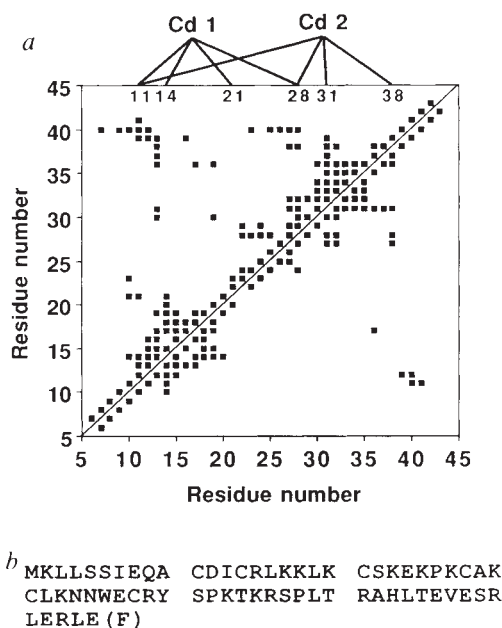


FIG. 1 **a**, Diagonal plot of inter-residue NOE crosspeaks. Points above the diagonal represent an NOE between any two protons. Below the diagonal, only backbone–backbone NOE crosspeaks are plotted. At the top the coordination of the cysteines to the two bound cadmium ions are indicated. **b**, Primary amino-acid sequence (single-letter code) for the DNA-binding domain of GAL4.

METHODS. NMR measurements were performed on GN-500, Bruker AMX-500 and Bruker AMX-600 spectrometers using 1.0–2.3 mM solutions of the protein in 0.2 M NaCl, 20 mM sodium phosphate, pH 7.0 at 25 °C. Protein was expressed by an *Escherichia coli* plasmid vector with an isopropyl thiogalactoside-inducible *tac* expression system⁶. The construct carried the first 65 residues of GAL4 with an additional Phe (F) introduced as a 66th residue by a cloning artefact. ¹¹³Cd GAL4(65) was prepared using zinc in the growth medium followed by extensive dialysis against excess ¹¹³CdCl₂. Owing to the stronger binding of cadmium²⁰, all zinc was replaced by ¹¹³Cd. Uniformly ¹⁵N-labelled GAL4 was produced by using ¹⁵NH₄Cl as the major nitrogen source in M9 minimal medium. Backbone resonance assignments for residues 6 to 43 were obtained from NOESY spectra recorded with 56, 150, 250 and 500 ms mixing times in H₂O and in D₂O solutions, from TOCSY spectra recorded with 40, 60 and 80 ms mixing times in H₂O and D₂O, and from double-quantum filter COSY data in D₂O, following standard procedures¹³.

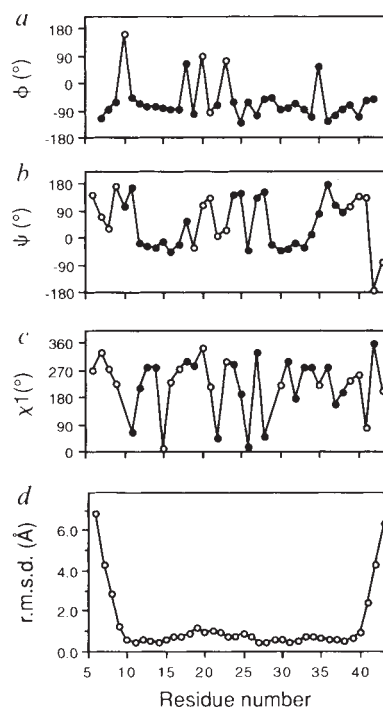


FIG. 2 Average torsion angles ϕ , ψ and χ_1 for residues 6–43 for 22 calculated structures of GAL4(65) and the average root-mean-square deviation (r.m.s.d.) of backbone atoms. Average torsion angles were obtained by adding two-dimensional unity vectors²¹ from all 22 structures (**a–c**). The length of the resulting vector is related to the standard deviation of the torsion angle²¹. Angles shown with filled circles have standard deviations of less than 30° and angles shown with open circles are less well defined. The backbone atoms of residues 9–40 for the set of structures presented in Fig. 4b were superimposed on the structure (Fig. 3) with the lowest distance violations. The average residual root-mean-square deviation over all possible pairwise fits is shown in **d**. Experimentally, torsion angles were inferred from the coupling constants measured using cross-sections of crosspeaks along ω_2 in two-dimensional spectra. For samples in D₂O solution, the α resonance of some residues showed only small coupling constants to both β protons for which the χ_1 torsion angle could therefore immediately be assigned as *gauche*[−] ($\chi_1 = +60^\circ$; residues 11, 22 and 28). Several others showed one strong and one weak coupling constant and could be distinguished as non-*gauche*[−] ($\chi_1 = -60^\circ$ or 180°). In some cases, NOE crosspeaks between the amide proton and the β protons allowed stereospecific assignment, and further characterization of the χ_1 angle as being either *gauche*⁺ or *trans*. The χ_1 angles of D12, K18 and N34 were further characterized through the ¹⁵N- β three-bond coupling constants^{22,23}. Constraints for ϕ dihedral angles were obtained from the HN-H α coupling constants. Definition of other torsion angles resulted from the interproton distance restraints imposed from the observed NOE crosspeaks (see Fig. 1).

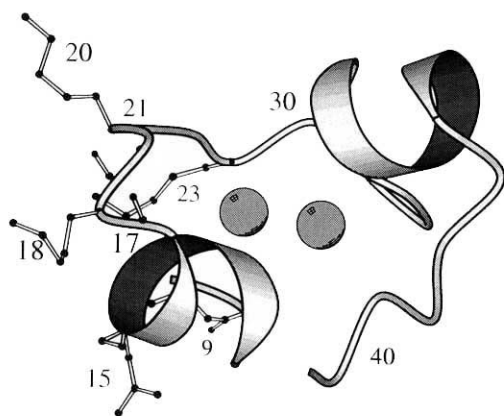


FIG. 3 Ribbon diagram representing the metal-binding module (residues 9–40) of the DNA-binding domain of GAL4. The amino-acid side chains (Gln 9, Arg 15, Lys 18, Lys 20, Lys 23), the carbonyl oxygens (Lys 17, Lys 18) and an NH group (Cys 21) that directly contact DNA are shown and numbered. The protein backbone is also numbered at residue 30 and 40 for reference. For NMR structure determination, crosspeaks in the 56 ms mixing time NOESY spectrum were quantified using the EASY program²⁴ and corresponding proton pairs were given lower and upper distance bounds. Calibration of NOE crosspeak intensities to interproton distances involved the use of the sequential α -proton to NH proton and the intrasidue NH to α proton crosspeak intensities^{21,25}. In NOESY spectra collected with longer mixing

times, crosspeaks were classified as being strong, medium or weak, and the corresponding protons were constrained with an upper bound of 3, 4 or 5 Å, respectively. Sulphur–cadmium liganding distances were imposed to be 2.35 to 2.45 Å, cysteine C β –cadmium distances were required to be less than 3.4 Å and distances between sulphurs liganding the same cadmium were set²⁶ to be between 3.3 and 4.2 Å. Hydrogen-bonds were implied between the amide protons of Cys 14–Lys 18 and Lys 33–Asn 35 and the carboxyl oxygen of the *i*-4th residue, by using lower and upper distance bounds of 2.0 and 2.8 Å, respectively. Within the compact domain (residues 9–40), the average r.m.s. deviation between any two aligned NMR structures is 1.4 Å for all atoms and ~ 0.6 Å for the main-chain backbone atoms. On solvating the protonated Zn(II) substituted GAL4(65) in D₂O at pH 7 and 25 °C for 2 h, 8 amide proton resonances remained. The residues most resistant to exchange were Cys 11, Asp 12, Cys 14, Arg 15, Leu 19, Cys 28, Cys 31 and Leu 32. Most of these residues were found in initial structure calculations to be part of α -helices with extensive α -N(*i*+3) and α - β (*i*+3) NOE connectivities justifying the use of the hydrogen-bond constraints. Strong NOE crosspeaks were observed between the α proton of Ser 41 and the δ protons of Pro 42, indicating that the ω bond is *trans*. The Lys 25 α –Pro 26 δ NOE was very weak, however, and a Lys 25 α –Pro 26 α NOE was observed instead, showing that Pro 26 has a *cis* peptide bond. The set of lower and upper distance bounds and torsion angles were used in the distance geometry program DG-II (ref. 17). Of the 25 structures calculated, 22 had residual error function values of 1.0 ± 0.2 kcal per mol. The other 3 had errors > 1.6 and were subsequently dropped for conformational analysis. Uniformly labelled ¹⁵N GAL4(65) has been prepared and is being used to complete the ¹H NMR assignments for the unstructured portion of the protein using three-dimensional techniques (J.D.B., T. Mau, V. Thanabal, and G.W., unpublished observations).

evolution of this structure involved duplication of a single zinc-binding element.

The bimetal-thiolate structure in the recognition module of GAL4 represents a new kind of DNA-binding structure with tightly bound zinc. Sequence similarity indicates that homologous structures are present in the fungal transcription factors Lac9 and PPR1. The zinc-finger, found in TFIIIA and many other transcription factors, and the steroid-receptor DNA-binding domain have been termed class 1 and class 2 zinc domains¹⁸ and we refer to the GAL4 module and its relatives as a class 3 zinc domain. There is no obvious similarity in the way each of these structures ligands the metal ions.

The structure of GAL4(65) in complex with a DNA containing the 17-base-pair consensus GAL4-binding site has been determined crystallographically¹². As expected, two monomers bind to the twofold symmetric site. The conformation of the compact

domain, residues 9–40, is nearly identical to the one described here. The r.m.s. deviation for the positions of backbone atoms, after superimposing the two structures, is 1.1 ± 0.1 Å (Fig. 4b). The metal-binding domain is a DNA recognition module, in the sense that it lies in the major groove with the right turn at the end of the first helix in contact with base pairs. Side chains (Gln 9, Arg 15, Lys 18, Lys 20, Lys 23) and backbone groups (CO Lys 17, CO Lys 18, NH Cys 21) that contact DNA are shown explicitly in Fig. 3. Residues 41–49 form an extended linker segment, connecting the recognition modules of each monomer to a dimerization element (residues 50–64) that lies over the centre of the DNA site. The principal dimer contacts are made by α -helical segments (residues 52–64) in a short coiled-coil structure. The NMR observations show that this dimerization element does not form when the protein is free in solution. But we do know that longer fragments, such as

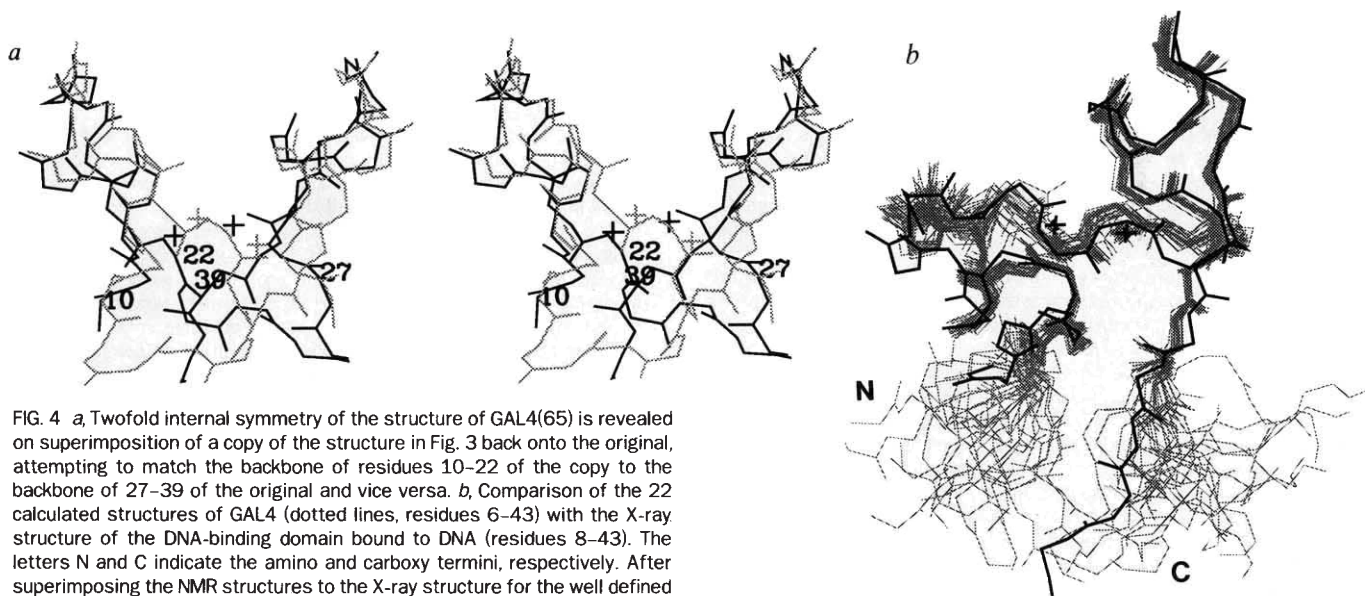


FIG. 4 *a*, Twofold internal symmetry of the structure of GAL4(65) is revealed on superimposition of a copy of the structure in Fig. 3 back onto the original, attempting to match the backbone of residues 10–22 of the copy to the backbone of 27–39 of the original and vice versa. *b*, Comparison of the 22 calculated structures of GAL4 (dotted lines, residues 6–43) with the X-ray structure of the DNA-binding domain bound to DNA (residues 8–43). The letters N and C indicate the amino and carboxy termini, respectively. After superimposing the NMR structures to the X-ray structure for the well defined globular cluster comprising residues 9–40, the r.m.s. deviation for the positions of backbone atoms is 1.1 ± 0.1 Å.

GAL4(1–100) and GAL4(1–147), are dimers even in the absence of DNA⁴ which has an additional dimerization element compared with GAL4(65)^{4,6,19}; GAL4(1–100), has a lower equilibrium dissociation constant (K_d) for binding UAS_G-containing DNA (R.M., M. Carey, S. Liang, M. Ptashne and S.C.H., unpublished data). Our picture of the longer fragment, and of intact GAL4, is therefore one in which two N-terminal recognition modules are connected by flexible linkers to a dimeric core. □

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Effect of alanine versus glycine in α -helices on protein stability

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THE rational design of proteins requires knowledge of the helix-forming propensities (s -values) of the different amino acids. There is, however, considerable controversy about the relative values for alanine and glycine^{1–12}. We find from experiments on mutants of barnase that the relative effect of Ala versus Gly on helix stability depends crucially on the position in the helix (whether they are at the ends (caps) or are internal) and the context (the influence of their neighbours). Glycine is greatly preferred at the N and C caps. At internal positions, Ala stabilizes the helix relative to Gly by 0.4 to 2 kcal mol⁻¹. The variation results from a combination of burial of hydrophobic surface on folding and interference with hydrogen bonding of the protein with solvent. There is a good empirical correlation between the relative stabilizing effects of Ala and Gly with the total change in solvent-accessible hydrophobic surface area of the folded protein on mutation of Gly to Ala. It is not valid to assign to each amino acid a unique s -value that is generally applicable to all positions in all helices in all proteins.

Several groups have examined the helix-forming propensities of various amino acids by the host-guest method^{1,2} or by making mutations in monomeric^{3–7,10} or dimeric helix-forming peptides⁸ and more complex systems¹¹. There are considerable discrepancies between measurements of the relative helix-forming propensities (helix propagation parameter, s)¹⁰ of alanine and glycine from copolymer host-guest experiments, analysed by the Zimm-Bragg formulation ($s_{\text{Ala}} = 1.07$, $s_{\text{Gly}} = 0.62$, equivalent to Ala stabilizing a helix by 0.3 kcal mol⁻¹ relative to Gly)¹³ and the more complex Lifson-Roig¹⁴ theory ($s_{\text{Ala}} = 1.56$, $s_{\text{Gly}} = 0.015$, equivalent to 2.8 kcal mol⁻¹ stabilization by Ala relative to Gly)¹⁰. (The change in stability of a protein on mutation of Ala to Gly at a particular position, $\Delta\Delta G_{\text{unf}}$, is related to the helix-forming propensities by $\Delta\Delta G_{\text{unf}} = -RT \ln(s_{\text{Ala}}/s_{\text{Gly}})$.) There can be problems in using model peptides because they are poorly characterized in solution. They are generally less than 100% helical and it is likely that the ends fray more than the middle^{8,10,11}. Different results are obtained when applying multistate or two-state models^{6,10}. Experiments on proteins are more amenable to interpretation because the parent structures are well defined and the helices are held intact

by the bulk of the protein. The derived experimental data may also be analysed by a simple two-state model.

Barnase is suitable model protein to study the effect of single mutations on protein stability^{15–21}. It has two regular α -helices (residues 6–18 and 26–34) which contain different residues and are partly exposed to solvent. Our strategy consists in selecting those residues that are on the solvent-exposed face of the helices and do not make extensive contacts with the rest of the protein. These residues are mutated to Ala and Gly, and the changes in protein stability ($\Delta\Delta G_{\text{unf}}$) measured with respect to the Ala mutants. In the first helix, we have selected the N-cap residue (Thr 6), Asp 8 and Asp 12, Thr 16, Tyr 17 and the C-cap residue (His 18). In the second helix, we have chosen the N-cap residue (Thr 26), Lys 27, Ser 28, Glu 29, Gln 31 and Ala 32, all of which make very few or no contacts outside the α -helix, and the C cap (Gly 34). The analysis of Ala $\rightarrow$ Gly mutations at the N cap has already been published¹⁵. The analysis of all the mutants is shown in Table 1. There is a clear-cut difference between the effects if the different mutations at the N cap, C cap and at internal locations. Gly is favoured over Ala at both caps (–0.5 or –0.9 kcal mol⁻¹ at the N cap; –1.2 or –3.1 kcal mol⁻¹ at the C cap). At all the other positions, Ala is favoured over Gly but there is considerable disparity in the differences in free energy of unfolding between Ala and Gly at the different internal positions.

The positional dependence of the relative helix-stabilizing propensities of Ala and Gly in the helices derived from small peptides in solution has been attributed to fraying at the ends¹⁰. The difference in α -helix propensity between Ala and Gly in these helices decreases monotonically towards the N or C caps but it never results in a higher stability for a peptide having Gly instead of Ala¹⁰. The results on barnase show clearly that there is a genuine positional and context dependence for the relative helix-stabilizing propensities of Ala and Gly that does not result from fraying. The data may be readily rationalized by simple physical principles that relate to solvent exposure (Table 2). First, the N and C Caps have exposed NH and CO groups, respectively, that require hydrogen-bond formation with either groups on the enzyme or by solvent^{22,23}. Bulky substituents at the N (ref. 15) or C caps that cannot satisfy the hydrogen-bond requirements of the first and last four residues of helices inhibit the solvation. Second, there is burial of hydrophobic surfaces on the formation of the helix, and this should be a stabilizing factor. It has been shown for small molecules that there is a linear relationship between free energies of transfer of hydrocarbon side chains from water to hydrophobic solvents and the change in accessible surface area^{24–27}. There is thus a physical reason for attempting to correlate hydrophobic effects with

changes in solvent-accessible hydrophobic area. We find that plotting $\Delta\Delta G_{\text{unf}}$ against ΔA_{HP} , the change in solvent-accessible hydrophobic area in the folded protein on mutation of Gly $\rightarrow$ Ala (Table 2), gives a good correlation (Fig. 1):

$$\Delta\Delta G_{\text{unf}} = 1.85 - 0.055\Delta A_{\text{HP}} \quad \text{kcal mol}^{-1} \quad (1)$$

The details of the calculation are given in Table 2; ΔA_{HP} = (total solvent-accessible hydrophobic surface area of mutant containing Ala – total solvent-accessible hydrophobic surface area of mutant containing Gly)_{folded protein}. The data for the N and C caps are excluded from this plot, as are also those for other positions where mutation of Gly $\rightarrow$ Ala buries exposed hydrophilic surfaces that require intermolecular hydrogen bonding (exposed backbone NH group at the N termini and the CO at the C termini plus surrounding side chains and main chain atoms; Table 2). (There is also a problem at the C cap of position 34 because Gly 34 in the native protein has ϕ - and ψ -angles that are energetically unfavourable for Ala and larger amino acids.) Note that the value of $55 \text{ cal mol}^{-1} \text{ \AA}^{-2}$ relating changes in energy to changes in surface area is some 2 times higher than found²⁷ from transfer free energies. This value is, however, consistent with extensive experimental data from other hydrophobic mutations^{28,29} and similar to new theoretical estimates³⁰.

We have also searched for correlations with changes in solvent-accessible hydrophilic area on mutation, although there is not precedence for this. Multiple linear regression for the variation of $\Delta\Delta G_{\text{unf}}$ with changes in both areas shows no significant dependence on the change in solvent-accessible hydrophilic surface area for the changes in internal positions in the helix. There is a correlation, however, if the only hydrophilic surfaces that are considered are those that have hydrogen-bond donors or acceptors that should make hydrogen bonds with solvent. Multiple linear regression for the variation of $\Delta\Delta G_{\text{unf}}$ with changes in solvent-accessible hydrophobic surface area and with

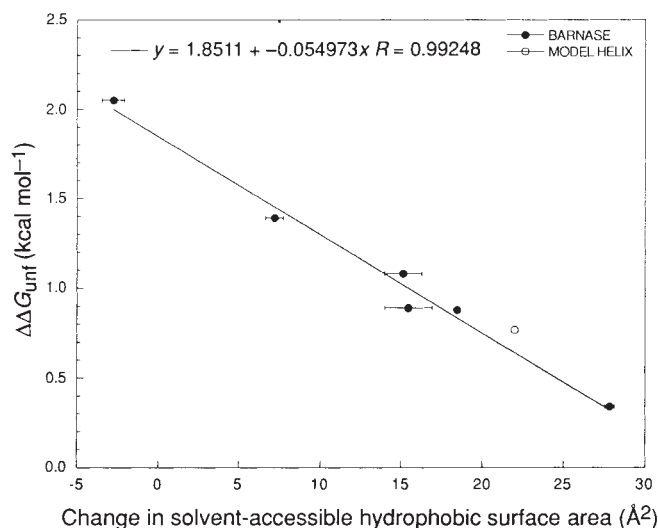


FIG. 1 Correlation between total changes in solvent-accessible hydrophobic surface area on mutation of Ala to Gly in the folded protein and the effect on protein stability. ○, Value for mutation of Gly to Ala found by O'Neil and DeGrado⁸ using surface-accessible areas calculated by modelling their peptide using Quanta and the Polygen suite of programs. The mutation of Gly $\rightarrow$ Ala is calculated to increase the solvent-accessible hydrophobic surface area by $22 \text{ \AA}^2$, which is close to that calculated for a model polyaniline helix ($21 \text{ \AA}^2$). The error bars represent the spread from averaging of data for the three molecules of barnase in the unit cell. The linear correlation holds only for the folded state. A simple thermodynamic analysis suggests that the correlation should be between $\Delta\Delta G_{\text{unf}}$ and the change in surface area of the folded state on mutation minus the change in that for the unfolded state. Presumably, the energy difference between Ala and Gly in the unfolded state of the protein is roughly independent of sequence and so is a constant term in the equation.

TABLE 1 Stability of barnase and mutants with Ala and Gly residues at different positions in the major α -helices

Position and residue in wild type	Change in energy on substitution of Ala $\rightarrow$ Gly ($\Delta\Delta G_{\text{unf}}$) in kcal mol ⁻¹
N cap (Thr 6)	-0.93
N cap (Thr 26)	-0.50
N + 1 (Lys 27)	0.76
N + 2 (Asp 8)	0.34
N + 2 (Ser 28)	0.86
N + 3 (Glu 29)	0.56
M (Asp 12)	0.88
C - 3 (Gln 31)	1.08
C - 2 (Thr 16)	1.39
C - 2 (Ala 32*)	0.91
C - 1 (Tyr 17)	2.05
C cap (His 18)	-1.15
C cap (Gly 34)	-3.12

The free energies of unfolding of wild-type protein and mutants were analysed by urea denaturation monitored by fluorescence¹¹⁻¹⁷. The data for a single mutant are reliable to $\pm 0.02 \text{ kcal mol}^{-1}$. The change in stability between mutant proteins with Ala or Gly at a certain position is obtained by subtracting the difference in stability between wild-type and an Ala mutant, from the difference in stability of wild-type and a Gly mutant. (–, Gly more stable than Ala.) Mutants and Proteins were prepared as previously described¹¹⁻¹⁷.

* Data from A. Horovitz and J. Matthews (unpublished results).

decrease in solvent accessible area of such groups (ΔA_{HB} ; Table 2) gives the correlation:

$$\Delta\Delta G_{\text{unf}} = 1.68 - 0.041\Delta A_{\text{HP}} - 0.19\Delta A_{\text{HB}} \quad \text{kcal mol}^{-1} \quad (2)$$

(where the standard errors for the three terms are ± 0.24 , ± 0.013 and ± 0.03 , respectively). The simpler equation (1) fits the internal residues slightly better, but there is a fair fit of observed values and those calculated by equation (2) (Fig. 2).

The absolute values for the helix-forming propensities of the different amino acids result from a variety of factors that change between the folded and unfolded states. For example, the backbone around glycine has a greater rotational freedom than for any other residue in the unfolded state, which favours unfolding³¹. It is clear, however, that the relative helix-forming propensities of Ala and Gly are strongly context-dependent, and one factor is changes in solvent exposure. When the Ala that is

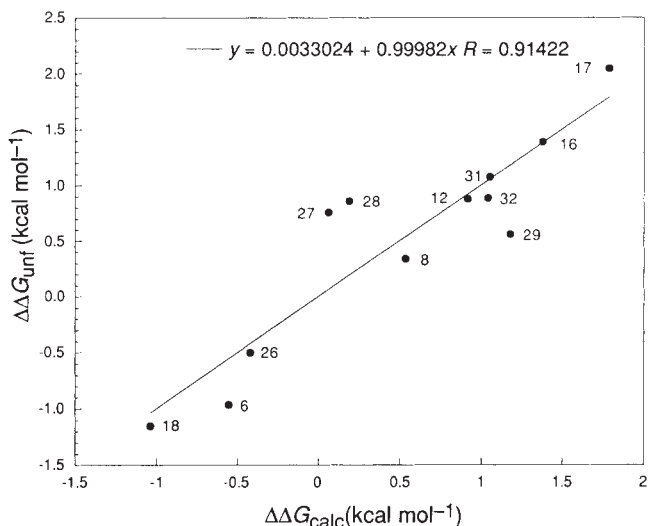


FIG. 2 Comparison of observed changes in free energy of unfolding on mutation of Ala to Gly and those calculated from equation (2) ($\Delta\Delta G_{\text{unf}} = 1.68 - 0.041\Delta A_{\text{HP}} - 0.19\Delta A_{\text{HB}}$) for the different positions in the helices.

TABLE 2 Changes in solvent-accessible area in folded protein on mutation of Ala → Gly at different positions in the two helices of barnase

Position	Increase in total solvent-accessible area		Decrease in solvent-accessible area of hydrophilic groups that require solvation† (ΔA _{HB}) (Å ²)
	Hydrophobic* (ΔA _{HP}) (Å ²)	Hydrophilic* (Å ²)	
6 (N cap)	22.4	-12.4	6.8
8	27.8	-13.5	0
12	18.5	-9.8	0
16	7.2	-4.1	0
17	-2.7	-3.7	0
18 (C cap)	15.8	-14.4	10.7
26 (N cap)	3.1	-10.0	10.2
27	7.3	-11.0	6.8
28	27.2	-8.6	1.9
29	3.7	-4.2	1.8
31	15.2	-6.6	0
32	15.5	-6.4	0

* The figures tabulated are the differences in solvent-exposed hydrophobic surface area in the folded protein having Ala instead of Gly. These were calculated using the program of Shrake and Rupley³³, as implemented by Miller *et al.*³⁴. The Ala or Gly mutants were constructed by deleting the surplus side-chain atoms from the coordinate file of barnase. Similar results were obtained using the program Quanta (Polygen) with which the target side chains were mutated to Ala or Gly using the protein design package of the problem. The exposed hydrophobic or hydrophilic area of the whole protein was calculated from the surface generated by the locus of the centre of a sphere of radius 1.4 Å rolling over the exposed van der Waals surface. The calculated solvent-accessible hydrophobic or hydrophilic area for the mutant containing Gly was subtracted from that calculated for the mutant containing Ala to give the value ΔA_{HP} or the hydrophilic equivalent.

† The changes in solvent-accessible area of non-intramolecularly hydrogen-bonded groups and hydrophilic groups of the protein, on mutation of Ala to Gly, were calculated for the folded protein using the program of Miller *et al.*³⁴. These are mainly for changes in the exposure of the backbone NH groups at the N termini of the helices, and the CO at the C termini, with some contributions from neighbouring side chains and backbone.

mutated is screened from solvent so that there is little change in solvent exposure on mutation to Gly, the helix is stabilized by some 2 kcal mol⁻¹ by Ala. At the other extreme, where the Ala is highly exposed to solvent, it stabilizes the helix by only some 0.4 kcal mol⁻¹, relative to Gly. We have applied equation (1) to a model polyaniline helix ($\phi = -65^\circ$ and $\psi = -41^\circ$). The predicted change in free energy of unfolding on mutation of Ala to Gly in an internal position of the α -helix is 0.7 kcal mol⁻¹. This is close to the experimental value found by O'Neil and DeGrado⁸ (0.77 kcal mol⁻¹) for an internal position in a helical peptide that has a change in solvent-accessible hydrophobic surface on mutation of Ala → Gly similar to that in a model polyaniline helix (Fig. 1) and at the extreme of the experiments by Kemp *et al.*¹¹ (0.17 to 0.77 kcal mol⁻¹). The peptide used by O'Neil and DeGrado⁸ is unlikely to fray, whereas that of Kemp *et al.*¹¹ is complicated by end-fraying. The positional dependence for the relative helix-forming propensities of Ala versus Gly may account for the high ratio of *s* values estimated¹⁰ from the Lifson-Roig theory, which gives a value of 2.8 kcal mol⁻¹ for the relative stability of Ala versus Gly in a polyaniline model helix instead of 0.7 kcal mol⁻¹. It was assumed in the calculations that *s* is independent of position in the model substituted-polyaniline helix and, implicitly, that the variation in apparent propensity is caused solely by fraying at the ends¹⁰.

Our empirical correlations show the importance of hydrophobic effects in protein folding and how they can influence helix-forming and, presumably, other structure-forming propensities of side chains, as suggested by Richards and Richmond³², and also the importance of solvent accessibility of

hydrogen-bonding groups²². It is not valid to assign to each amino acid a unique *s* value that is generally applicable to all positions in all helices in proteins. □

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Mapping antibody binding sites on protein antigens

Yvonne Paterson

The large surfaces of protein antigens that interact with antibody-combining sites can be determined using deuterium-exchange labelling and two-dimensional ^1H nuclear magnetic resonance (NMR). This technique may also be applied to other protein-protein interactions to identify key residues that contribute to the affinity.

THE availability of epitope-specific monoclonal antibodies produced by hybridoma technology has resulted in an explosion of uses for these products in medicine and industry. However, in the case where the antigen is a large protein molecule, the identity of the surface on the antigen that is recognized by the antibody, and the nature of the forces that stabilize the interaction, are still largely unknown. This is because the methods available to identify the epitopes of protein antigens are very limited. Most of what we know about the interface between the antibody-combining site and antigenic sites on proteins has been derived in the last few years from X-ray crystallographic studies on five immune complexes of the Fab fragments of monoclonal antibodies and two protein antigens, hen egg lysozyme and influenza neuraminidase¹⁻⁴. These studies revealed that an extensive surface of the protein antigen, about 700–900 Å, comprising two to four discontinuous stretches of polypeptide backbone, interacts with the antibody-combining site⁵.

The ability to study antibody/antigen complexes at the atomic level allows us to explore the nature of the interaction and the degree of conformational change that takes place in either the antibody or the antigen on complex formation. As both neuraminidase⁶ and lysozyme⁷ have been crystallized in the free form, direct comparisons can be made between the antibody-bound and free forms of these antigens. Differences in the degree of complementarity have been observed in these existing antibody/antigen structures. However, the existing antibody/protein antigen structures provide no concrete evidence on whether conformational changes can also take place in the antibody on interaction with a protein antigen as, at this point in time, a detailed structural comparison of the complexed and uncomplexed immunoglobulin molecule has not yet been performed. We believe

this will not be too far in the future. One of the anti-lysozyme antibodies has now been crystallized in the free form and its structure is currently under investigation in Roberto Poljak's laboratory⁸. In addition, Elizabeth Getzoff and co-workers at Scripps Clinic, and myself are also in the process of solving the structure of an anti-horse cytochrome *c* Fab fragment (E8) in the free and complexed form⁹. The structure of D1.3 and E8 in comparison with their structures complexed with protein antigens, should resolve this important issue.

Immune complexes in solution

The first structural determination of an antibody complexed with a protein antigen was solved in 1986 by Roberto Poljak and co-workers¹. In the five years since then, only a few more antibody/protein antigen crystal structures have been published. This indicates the difficulties inherent in the application of X-ray

crystallography to the large molecular complexes formed by protein antigens with their antibodies. Thus, although X-ray crystallography provides the most detail at the molecular level about the surface on a protein antigen that is occluded by antibody binding, it is limited by problems in preparing homogeneous enzymatically-cleaved Fab products of antibodies and in preparing crystals of immune complexes that are suitable for X-ray diffraction. In addition, even when sufficient X-ray data at high resolution are obtained, the interpretation of the data for such large structures to provide X-ray coordinates can take years.

For several years, my laboratory has been interested in developing biochemical techniques to map discontinuous antigenic sites on protein antigens that may be more generally and rapidly applied than X-ray crystallographic techniques^{10,11}. Perhaps the most successful approach has been to probe the surface of

the protein antigen that is protected by complex formation with a monoclonal antibody. Over the years, the probes that we, and others, have used to identify immunoprotection have included proteolytic enzymes such as trypsin or chymotrypsin¹⁰ or chemical reagents that modify specific functional side-chains of amino acid residues¹¹. However, these probes are too specific to identify more than one or two of the discontinuous regions of the polypeptide chain on the protein antigen that interacts with the antibody^{10,11}. Recently, we have exploited the ability of deuterium to exchange with amide protons in the polypeptide backbone of a protein to provide us with a more generic probe; one that has the potential to identify every amino acid residue, except proline, in a protein molecule.

Deuterium exchange with the protons of a protein results in this atom becoming undetectable in an ^1H NMR spectrum. For amide protons, this is manifested by a reduction of peak area for a one-dimensional spectrum or a re-

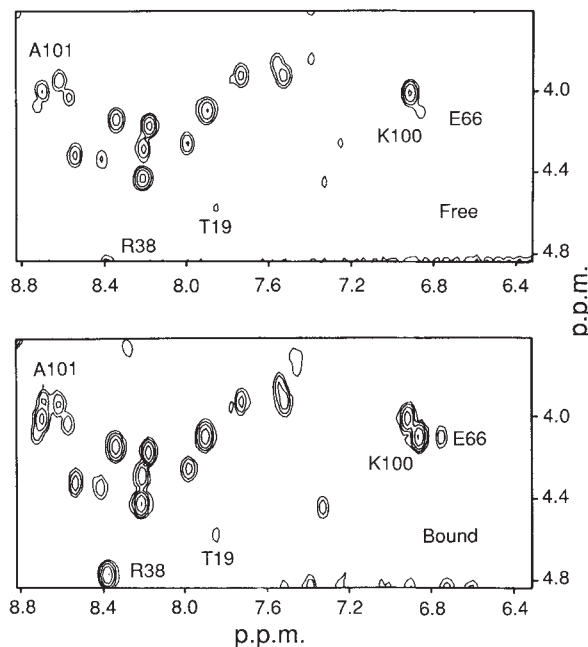


FIG. 1 Sections of two-dimensional J -correlated ^1H NMR spectra recorded after nine hours of D_2O exchange with free cytochrome *c* and cytochrome *c* bound to the monoclonal antibody E8. Note that the cross-peak volumes from the residues R38, E66, K100 and A101 are visibly different, whereas the cross-peaks from other residues in cytochrome *c*, for example, T19, are unaffected by antibody binding.

duction of cross-peak volumes in the J -correlated $\text{NH-C}\alpha\text{H}$ region of a two-dimensional (2D) spectrum (see Fig. 1). This technique has been used with great success to define those amide protons that become protected against exchange during the refolding of horse cyt c ¹². In our application, we measured the rate of hydrogen-deuterium (H-D) exchange with the amide protons of an antigen/antibody complex of horse cyt c and a monoclonal antibody (E8), and analysed those protons protected by complex formation using 2D-COSY NMR¹³. We found that the H-exchange rate of residues in three discontinuous regions of the cytochrome c polypeptide backbone is slowed by 7 to 340 fold in the antibody-antigen complex compared with free cytochrome c . The protected residues, 36–38, 59–67 and 100–101, and their H-bond acceptors, are brought together in the three-dimensional (3D) structure on one surface of the protein. Defined in this way, the interaction site¹³ is consistent with prior epitope mapping studies on the antibody^{10,11} and the size of the molecular surface on cytochrome c which would be occluded by interaction with antibody, about 750 Å, is comparable to that determined by X-ray crystallographic analysis of other monoclonal antibodies complexed with small protein antigens⁵. In addition, the variation in the magnitude of the protection factors indicates that the amide protons of some residues can exchange with deuterium only when the antigen is dissociated from the antibody, suggesting that these residues are crucial in stabilizing the interaction, whereas others clearly exchange while still in the complex.

A further advantage of using a solution structural method, such as NMR, to examine antibody/antigen interactions, is that it might provide a more dynamic view of the epitope, one that is more representative of physiological conditions than that provided by X-ray crystallography. In general, however, ¹H NMR spectroscopy is not directly applicable to antibody/protein antigen complexes due to the large number of inter-proton interactions generated by such large molecular complexes. We solved this problem by immobilizing the antibody on a solid support, which allows us to analyse the antibody/antigen interface using H-D exchange labelling and then to dissociate rapidly the antigen (see Fig. 2). Using this technique, it is possible to perform 2D NMR spectroscopy on the protein antigen alone, in isolation from the antibody¹³.

Future applications

The H-exchange labelling method has many advantages over other methods for 3D epitope mapping. Although it lacks the resolution of X-ray crystallography,

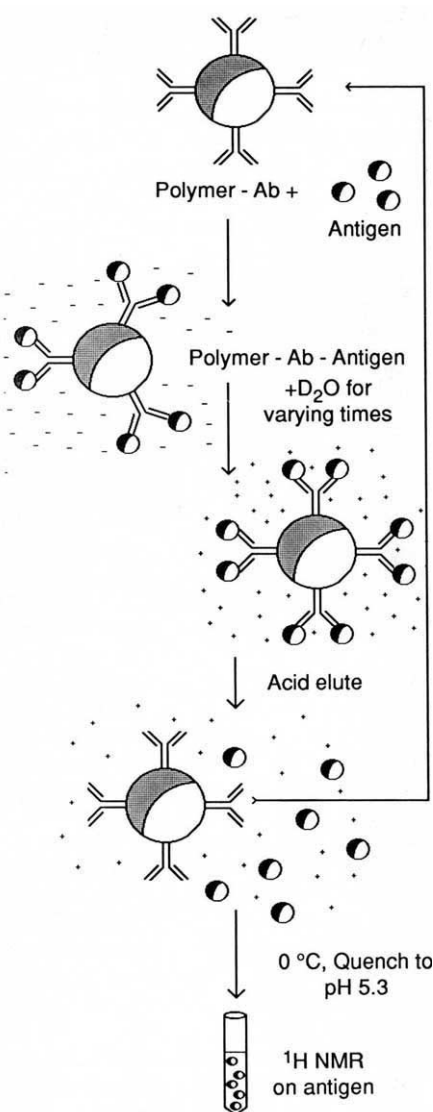


FIG. 2 Method for hydrogen exchange on an antibody-bound protein antigen in preparation for analysis by two-dimensional NMR in the absence of the antibody. Antibody (Ab) is covalently coupled to a solid polymeric support before binding antigen.

in the example described above many more residues in the buried epitope were identified than with all of the other methods used together¹¹. The exchange reaction is non-invasive and unlikely to produce global conformational changes in the complex, and it is a non-destructive technique in that both the immobilized antibody and exchanged antigen can be re-exchanged with water and used in other experiments. The structures of an increasingly large number of small proteins (less than about 150 residues) are being solved by 2D-NMR spectroscopy. Over 100 small proteins have assigned NMR spectra and the solution structure has been determined for about half of them (reviewed in ref. 14). We anticipate, therefore, that this method will be invaluable in studies on the antigenicity of small proteins, and may also be applied to protein-protein interac-

tions in general. A particularly appropriate application of the H-exchange labelling procedure would be to identify residues in the buried interface between a small protein ligand and an immobilized receptor, which are highly protected from H-D exchange, to target for site-directed mutagenesis in studies aimed at improving the affinity of the interaction. □

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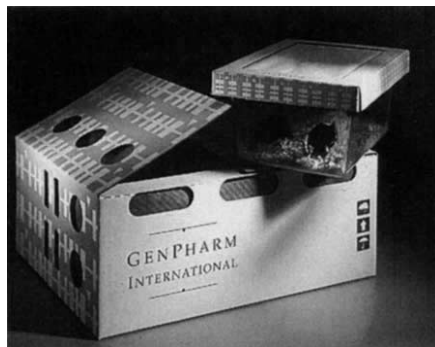
Eye on the life science marketplace

Over 400 companies will be exhibiting at next week's meeting of the Federation of American Societies for Experimental Biology to be held in Anaheim, California. A new p53-deficient transgenic mouse strain and a laser-induced fluorescence detector for capillary electrophoresis will be featured.

In booth 814 at FASEB, PharMingen will be exhibiting BaculoGold, an easy-to-use **baculovirus transfection kit** (*Reader Service No. 101*). It features, the company says, 100 per cent recombination efficiency and eliminates the need for plaque assays. The BaculoGold transfection kit includes a novel, linearized baculovirus DNA containing a lethal deletion, a complementing rescue plasmid, and a baculovirus instruction manual. Also available is a full line of single- and dual-promoter baculovirus transfer vectors, unique virus DNAs, and insect cell lines. PharMingen also offers custom baculovirus services.

Multiple Peptide Systems has introduced a **mass spectral analysis service** for peptide samples (*Reader Service No. 102*). This analysis, which MPS already performs on all peptides that it custom synthesizes, is now being offered for peptides synthesized by researchers. The company uses a BIOION 20 time-of-flight mass analyser to assure the identity of a synthetic peptide by verification of the exact molecular weight. The mass spectral analysis service can produce spectra in an extremely high mass range and provide assured mass accuracy, MPS says. The company, which guarantees a 48-hour turnaround time for samples, will be in booth 1642.

A new TSG-p53 strain of **transgenic mouse** completely deficient in the p53 tumour suppressor gene is being offered for sale to researchers by GenPharm, which has acquired exclusive worldwide rights to the mouse from Baylor College of Medicine (*Reader Service No. 103*). As mutations in the p53 tumour suppressor gene are the most frequently observed genetic mutation in human cancers, GenPharm hopes these mice will provide useful models for screening chemotherapeutic agents and for investigating carcinogenesis. Researchers, led by Lawrence Donehower and GenPharm scientific advisor Allan Bradley eliminated the p53 tumour suppressor gene's function by introducing a null mutation into that gene in embryonic stem cells, using a technique known as homologous recombination. Mice that carry the p53 deficiency in both sets of chromosomes (homozygous mice) exhibit dramatic early onset of a broad spectrum of malignant tumours, including lymphomas, sarcomas and carcinomas. GenPharm says that this



GenPharm's p53-deficient transgenic mice.

tumour profile is somewhat suggestive of the tumour susceptibility in Li-Fraumeni syndrome, an inherited human cancer susceptibility syndrome associated with p53 mutations. In contrast, heterozygous mice (that is, mice that carry only one copy of the p53 deficiency) show a low spontaneous level of tumorigenesis. GenPharm will be in booth 1638.

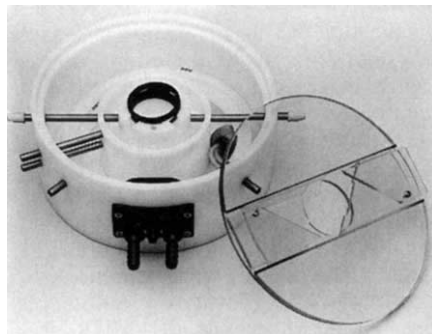
With the scientific and medical community in mind, Polaroid has developed an **instant colour print film for use with tungsten lighting** (*Reader Service No. 104*). Polacolor 64 Tungsten is designed for such scientific and medical applications as photomicrography and photomacrography. With an ISO rating of 64 and a 90-second development time, the new film is optimized for longer duration exposures, eliminating the need for colour-correction filtration used in converting daylight-balanced films for tungsten lighting. Polacolor 64 Tungsten uses a new negative and modified developing reagent chemistry, which is intended to provide higher contrast and colour saturation. Available next month, the film will be offered in the following formats: 3.25 × 4.25-inch pack film and 4 × 5-inch sheet film. Other formats will be added later this year. Polacolor 64 Tungsten is balanced for 3,200 K illumination, with an optimal no-correction exposure of four seconds. In many applications, however, Polaroid says it requires no filtration for exposures from 0.5 to 32 seconds. See booth 2141.

Onscreen graphics editing, better fonts, a capacity for larger data sets, user-defined curve-fitting equations and support for colour printers are some of the features that version 4.0 of InPlot, **software for scientific graphing and data analysis** from GraphPad, offers over the

earlier version, 3.1 (*Reader Service No. 105*). InPlot's features include curve fitting using nonlinear regression, mathematical transformation of x or y values, linear regression, calculation of mean and error bars from replicate values, and the ability to handle data with missing values. Versatile graphics allow the user to move or re-size any portion of the graph (titles, labels or a complete graph), plot any number of lines or curves on a single set of axes, plot data on logarithmic or discontinuous axes, and create histograms and bar graphs. InPlot also supports a complete set of scientific and international characters. InPlot version 4.0 for IBM PCs and compatible computers costs \$395 (US). See booth 2247.

Tissue/cell culture aids

InVitro-1 is a small **benchtop environmental chamber** from World Precision Instruments designed for keeping eukaryotic tissue alive for several days *in vitro* during electrophysiological experiments (*Reader Service No. 106*). Although InVitro-1 was designed primarily to study



Environmental chamber for *in vitro* tissue from World Precision Instruments.

single nerve-cell activity in a thin slice of mammalian central nervous system tissue, it can be used to maintain any tissue in a viable state for extended periods of time. Tissues can be of plant or animal origin and can range in complexity from simple multi-cell, single-layer organisms to complex three-dimensional organ systems. The chamber consists of two concentric rings surrounding a central chamber, allowing for control and rapid manipulation of the temperature and gas content of the perfusion medium, while simultaneously monitoring temperature, pH and dissolved oxygen. Temperatures within InVitro-1 are regulated by an external water bath. The thermal load of the

bath is transduced into the chamber through a heat exchange system in such a manner that the temperature of the entire chamber can be controlled and manipulated. The outer ring of the chamber is also used to produce humidified gas (typically 95 per cent O₂ and 5 per cent CO₂) by bubbling dry gas through a pool of free-standing water. The chamber can be autoclaved. See booth 1607.

With the new CO₂ water-jacketed **cell-culture incubator** from Forma Scientific, which incorporates the latest in fuzzy logic technology, just one model is available for all world electrical voltages (*Reader Service No. 107*). A door-mounted control panel features a microcomputer with vacuum fluorescent alpha/numeric display. The incubator is programmed using the touch keypad. Self-prompting messages allow the operator to control and monitor the performance parameters of the incubator. To reduce the possibility of contamination, the incubator features a robotic-welded, crevice-free, stainless-steel interior with coved corners. HEPA filters are easily accessible. See booth 1602.

DNA details

Make lightweight of DNA synthesis with the new Cyclone Plus **nucleic acid synthesizer** from Millipore (*Reader Service No. 108*). Cyclone Plus can be used to produce polymerase chain reaction or sequencing primers, probes and linkers (even long fragments). In a day, Millipore says it is possible to synthesize, deprotect and purify your oligonucleotides. First, load your reagents. Second, insert A, C, G, T and any unusual bases that your synthesis may require. (Prepackaged kits from Millipore are designed to make this a fast and fail-safe operation.) Then, key in your sequence and push 'run'. Expedite monomers, which are soon to be introduced by Millipore, are intended to shorten the processing time considerably. See booth 1022.

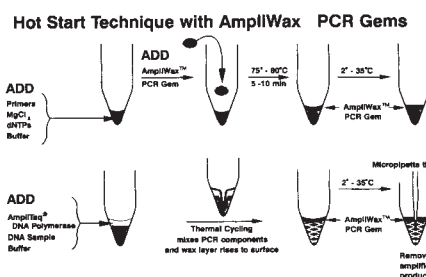
Beckman's new **laser-induced fluorescence (LIF) detector** for its P/ACE capillary electrophoresis system is designed to provide a 500–1,000-times increase in sensitivity as compared to UV



Laser-induced fluorescence detector for Beckman's capillary electrophoresis system.

detection (*Reader Service No. 109*). The LIF system includes the LIF detector, which fits onto the P/ACE unit, and a separate argon ion laser. The LIF system is designed to accommodate various types of work; the detector can be linked to a variety of types of laser, emission filters can easily be interchanged, and LIF and UV detectors can be exchanged. As a safety measure, special interlock devices ensure that the laser is turned off in the event of disconnection of the fibre-optic cable or removal of the detector. To save space, Beckman supplies a lengthy cable to allow the user to place the laser in a remote location. A P/ACE capillary electrophoresis system with built-in UV and LIF detectors costs \$55,000–\$65,000 (US). However, existing users of a P/ACE system can add the LIF detector for less than \$20,000 (US). Beckman's booth is 822.

AmpliWax PCR Gems from Perkin-Elmer have been designed to **replace mineral oil as a vapour barrier in polymerase chain reaction (PCR)** amplifications and to facilitate advanced PCR methods such as the 'hot-start' technique (*Reader Service No. 110*). Automation of the hot-start technique involves dispensing one AmpliWax PCR Gem into



AmpliWax PCR Gems — designed to replace mineral oil in PCR amplifications.

a tube containing some of the components of the GeneAmp PCR process (primers, dNTPs, MgCl₂ and buffer), heating and cooling the tube to melt the PCR Gem, and then creating a solid wax layer above a lower reagent mix. The remaining reagents (DNA template, AmpliTaq DNA polymerase and buffer) are then aliquoted on top of the barrier. In the first denaturation step of the GeneAmp process, the wax layer melts, the upper reagents mix thoroughly with the lower reagent mix, and the DNA is denatured. Preventing premature primer extension from initiating until the reagent mixture is hot avoids pre-PCR mispriming and primer oligomerization. According to Perkin-Elmer, this results in increased PCR specificity, yield and precision, especially when amplifying low copy number targets such as human immunodeficiency virus. After PCR amplification, the AmpliWax PCR Gem can be punctured with a pipette tip to withdraw the reaction mixture for post-

PCR analysis. The need to extract mineral oil with chloroform is thereby eliminated. See booth 1511.

For **separating large fragments of DNA using pulsed-field electrophoresis** technology, Hoefer recommends its new HG 1000 Hula gel horizontal unit (*Reader Service No. 111*). The Hula gel apparatus,



Hoefer's Hula gel apparatus.

which consists of a single set of stationary electrodes and a rotating gel platform, uses a technique known as 'rotating gel electrophoresis'. Hoefer says that the single set of electrodes ensures that the electric field is uniform, resulting in straight lanes with no distortions. Unlike other commercially available units that have a preselected, fixed reorientation angle, the Hula gel system (with its user-programmable microprocessor) allows the user to vary the reorientation angle from 0° to 360°, in one-degree increments. This ability to vary the angle allows the user to optimize the resolution and run time for each sample type. The small housing contains a built-in 300 V, 300 mA power supply, buffer recirculation coils and pump, a heat exchanger and a microprocessor. See booth 1522.

Dynal's Dynabeads Oligo (dT)₂₅ allow **production of reusable subtracted cDNA probes** for simplified screening of differentially expressed genes and for isolation of rare messages (*Reader Service No. 112*). Poly A⁺ RNA from one cell type is isolated with Oligo (dT)₂₅ in 15 minutes. Direct cDNA synthesis of the mRNA template extends from the oligo-dT primer present on the magnetic beads. Following elution of the mRNA template, the cDNA probes bound to the magnetic beads are then hybridized to mRNA of a second cell type. Unique mRNA sequences are then captured with a second aliquot of Oligo (dT)₂₅ and cDNA synthesis results in subtracted cDNA probes. Dynal says subtractive hybridization with Oligo (dT)₂₅ is a rapid method for isolation of cDNA corresponding to very rare mRNAs of a specific cell type. Subtracted cDNA probes bound to magnetic beads can be stored and reused to screen multiple libraries. See booth 434.

Assorted monoclonals

Telios Pharmaceuticals has introduced an extensive line of **monoclonal antibodies to human integrins**, which should provide useful research tools for use in ELISAs, immunofluorescence, immunoblotting, immunoprecipitation and cell attachment studies (*Reader Service No. 113*). The line-up includes antibodies to the $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, αV , $\beta 2$ and $\beta 4$ subunits. Additional antibodies are in development. See booth 1836.

A range of **PY20 mouse anti-phosphotyrosine reagents** are now available from Zymed Laboratories (*Reader Service No. 114*). These monoclonal antibody preparations are intended to provide the researcher with a useful set of tools for characterizing proteins involved in signal transduction and mediation of oncogene activity. PY20 has a very high affinity for tyrosine-phosphorylated protein but does not react with serine or threonine-phosphorylated proteins. It is an IgG_{2b} isotype and binds strongly to Protein A or Protein G. Zymed supplies the antibody either in a purified form or conjugated to fluorescein isothiocyanate, horseradish peroxidase, biotin or agarose. Stated applications include immunoprecipitation, immunofluorescence, western blotting and ELISAs. See Zymed in booth 708.

Sigma Immunochemicals, who will be exhibiting in booth 627, offers an extensive range of **human and mouse growth factors/cytokines and antibodies to growth factors** (*Reader Service No. 115*). Growth factor sources are natural, recombinant or synthetic, and all reagents undergo performance testing and quality control procedures. Sigma says it guarantees the biological activities of these growth factors. Growth factors and antibodies to growth factors are applications-tested to ensure performance, and each product is provided with a data sheet, which includes references, bioassay data and information on working dilutions. Listings include: epidermal growth factor

(EGF), fibroblast growth factors (FGFs), colony-stimulating factors (CSFs), interleukins (ILs), nerve growth factors (NGFs), stem-cell factor, platelet-derived growth factors (PDGFs), transforming growth factors (TGFs) and tumour necrosis factors (TNFs). Antibodies include: EGF, EGF-receptor, FGF, NGF, phosphotyrosine, PDGF β -receptor and TNF.

Harlan Bioproducts for Science is distributing Serotec's large selection of **monoclonal antibodies to CD antigens expressed on human haemopoietic cells** (*Reader Service No. 116*). With each vial of anti-CD monoclonal antibody purchased, the company supplies a vial of erythrolyse, Serotec's lysing buffer. Erythrolyse is used for lysing human red blood cells after immunofluorescence staining of cells in whole blood with Serotec's monoclonal antibodies. To help researchers with the increasing number of identified human CD antigens, Serotec has developed a wall chart (free on request) that describes the differentiation of human haemopoietic cells, depicts the subdivision of each lineage, and lists each CD antigen found on each cell type. See booth 939.

QUICKMeasure from Sterogene Bioseparations provides a rapid means of **determining the concentration of monoclonal antibodies** in cell culture or during purification (*Reader Service No. 117*). It can be used to determine the concentration of any class or subclass of antibody, including IgG, IgM, IgA and IgE, in about 30 minutes. According to Sterogene, the sensitivity limit of the assay is 1 μ g of antibody. If serum supplements are used in the culture medium, bovine immunoglobulin does not interfere with QUICKMeasure. The kit can be used to optimize cell culture conditions and monitor/optimize purification conditions. The assay is non-ELISA based and requires only a spectrophotometer to determine the results. See booth 2116.

Reagent roundup

Amersham has introduced 12 new Biotrak **non-isotopic assays for cytokines** designed to offer high sensitivity, specificity and convenience (*Reader Service No. 118*). Based on an immunometric sandwich assay, Amersham's non-isotopic cytokine assays can be used to measure the full range of cytokines in culture media, serum and plasma. The range presently includes interleukin-1 α (IL-1 α), IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, tumour necrosis factor- α (TNF α), TNF β , granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor. The assays use specific monoclonal antibodies coated onto a microtitre strip, which capture any cytokine in the sample. After washing, a second antibody labelled with peroxidase is added. Immobilized enzyme is quantified following the addition of a safe and sensitive tetramethylbenzidine/hydrogen peroxide substrate. Assays can be carried out in five hours. See Amersham in booth 1402.

Phthoxazolin, a new **inhibitor of cellulose biosynthesis**, can now be obtained from Kamiya Biomedical (*Reader Service No. 119*). Isolated from cultures of *Streptomyces* sp., phthoxazolin (OM-5714) should be a useful new tool for the study of cellulose biosynthesis in bacterial, fungal and plant systems. It inhibits the incorporation of ¹⁴C-glucose into extracellular cellulose fraction of *Acetobacter xylinum*. It also inhibits cellulose biosynthesis in a cell-free system from *A. xylinum*. In addition, phthoxazolin inhibits growth in a limited genera of fungi, including *Phytophthora* sp., which are known to contain cellulose in their cell wall. Kamiya Biomedical will be in booth 1227.

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